IMMUNOLOCALIZATION OF THE TWO SUCROSE SYNTHASE ISOZYMES IN MAIZE (*Zea mays* L.)

BY
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The expression of Sh and Sus genes, which encode sucrose synthase isozymes SS1 and SS2, respectively, was examined in maize kernels and roots by immunocytochemical techniques using light and electron microscopy. The spatial/temporal distribution of the two sucrose synthase isozymes was observed in different parts of kernel and root.

The two isozymes were differentially localized in developing endosperm cells through the combined uses of a shrunken (sh) mutant lacking the SS1 protein, a Sus-null mutant lacking the SS2 protein, and SS1 and SS2 antisera. The accumulation of SS1 protein coincided with starch deposition in the Sh endosperm cells; whereas the SS2 specific cells, including the aleurone layer and basal endosperm transfer cells in both Sh and sh genotypes, were not associated with detectable starch deposition. Intracellular immunolocalization of the two SS proteins in

immature endosperm revealed that the SS proteins were dispersed in the cytoplasm in Sh and sh genotypes; thus, no specific localization to cell wall or any organelle was detected.

In sh developing endosperm, the center of the crown region formed a cavity at or during the most critical phase of starch biosynthesis. The degeneration of the cell wall was observed using light and transmission electron microscopy; these observations were further elaborated using scanning electron microscopy.

In root sections, higher levels of sucrose synthase isozymes were localized in the vascular cylinder and epidermal cells than in the cortex. The intra-cellular pattern of SS protein distribution in root cap cells was found to be similar to that in endosperm cells, as the SS proteins were localized throughout the cytoplasm.

The previous demonstration of anaerobic (AN) induction of the Sh but not the Sus gene was also examined immunohistologically on root sections from aerobic and AN grown seedlings. A slight but consistent increase in SS protein was found in the root tip and root cap of the AN roots of Sh genotype. The Sus gene was also induced in the AN root cap cells of sh seedlings.

INTRODUCTION

In the maize plant (Zea mays L.), the endosperm serves primarily for starch storage and makes up about 85% of the kernel weight. Due to the starchy endosperm, maize is an important starch source for livestock feed and industrial utilization.

In the maize endosperm, sucrose is imported from photosynthetic tissues and is converted into starch in amyloplasts. The starch is synthesized using ADP-glucose or UDP-glucose as a sugar nucleotide donor. Both ADP-glucose and UDP-glucose are synthesized by the pyrophosphorylase reaction or by reversal of the sucrose synthase reaction (for review see: Goodwin and Mercer 1983; Akazawa and Okamoto 1980). The enzyme sucrose synthase (EC 2.4.1.13) catalyzes the following reversible reaction: Sucrose + UDP <---> UDP-glucose + Fructose. A positive correlation between the level of sucrose synthase activity and starch content in maize endosperm indicated that the role of sucrose synthase in storage tissue is the cleavage of sucrose (Pressey 1969; Tsai et al. 1970). This was further demonstrated by genetic evidence (Chourey and Nelson 1979). Many maize mutants cause starch deficiency in the endosperm and are identifiable due to shrunken or

shrivelled phenotypes of the kernel. The shrunken 1 (sh) mutant is one of the starch deficient mutants and is characterized by the shrunken kernel which contains about 60% of the starch found in the wild-type Sh kernel. A protein was identified in Sh1 endosperm extract, which was missing in extracts of homozygous recessive (sh1) endosperm (Schwartz 1960). Chourey and Nelson (1976) showed that the protein encoded by the Sh locus of maize is sucrose synthase. In addition, a residual sucrose synthase activity less than 10% of the sucrose synthase activity found in normal Sh endosperm, was detected in sh endosperm (Chourey and Nelson 1976). This residual sucrose synthase activity found in endosperm extracts of various shrunken mutants (Chourey 1981 a,b) was shown to be due to a second sucrose synthase isozyme.

The two sucrose synthase isozymes in maize, designated as SS1 and SS2, are encoded by the Sh and Sus genes, respectively. The SS1 protein is present in endosperm and root of the wild-type Sh genotype (Chourey et al. 1986) and constitutes a critical link in starch biosynthesis in the developing endosperm (Chourey and Nelson 1976, 1979). The expression of the Sh gene exhibits endosperm-specific abundance and is not detected in the embryo of the immature kernel (Chourey and Nelson 1976; Chourey et al. 1988). The complete absence of SS1 protein, as in certain sh mutants, is associated with a starch-deficient endosperm phenotype; no other plant part is phenotypically affected by the sh

mutation. Previously, it has been shown that expression of the Sh gene in roots is responsive to anaerobic stress. The Sh gene responds with a marked increase in RNA levels (Springer el al. 1986; McCarty et al. 1986) but no apparent increase in protein levels (McElfresh and Chourey 1988). The SS2 protein is present in endosperm, embryo and roots of Sh and sh genotypes. The Sus locus does not exhibit endosperm-specific abundance (Chourey 1981a; Chourey et al. 1988); and from studies using whole root extracts of RNA and protein, it does not appear responsive to anaerobic stress ((McCarty et al. 1986; Springer et al. 1986; McElfresh and Chourey 1988).

Another observation of much interest is the tissue specific polymerization of the SS1 and SS2 subunits (Chourey et al. 1986). Immuno-blot analyses of the Sh genotype reveal five SS isozymes comprised of two homotetramers and three heterotetramers in root extracts but only the two homotetramers in endosperm extracts. The lack of heterotetramers has been attributed to a spatial/temporal separation of expression of the two sucrose synthase genes in endosperm cells (Chourey et al. 1986).

At the molecular level, both Sh and Sus genes have been cloned and extensively characterized (Werr et al. 1985; McCarty et al. 1986; Gupta et al. 1988); however, little is known concerning the expression of these two genes at the cellular level. In order to better understand

the expression of the two sucrose synthase genes at the cellular level, immunocytochemical techniques with light and electron microscopy have been used to localize SS1 and SS2 proteins in the kernels and in aerobic and anaerobically stressed roots.

Data presented here describe the spatial and/or temporal expression of Sh and Sus genes in developing kernels and aerobic and anaerobically stressed roots of young seedlings. Intracellular localization of the two SS isozymes in the endosperm and root cells showed that SS proteins are distributed throughout the cytoplasm and not specifically on the cell walls or amyloplasts. In addition, the formation of a cavity in the endosperm of sh mutants is described.

Results from immunostaining aerobic and anaerobic stressed roots indicate that anaerobic stress induces the expression of the two sucrose synthase genes at the protein level. SS1 is induced in the root tip approximately in the lower 1 cm of the AN Sh root. SS2 is also induced in the root cap of AN sh root.

REVIEW OF LITERATURE

Maize Kernel

The maize kernel is a one-seeded fruit, in which the seed, consisting of embryo, endosperm, seed coats and nucellar remnants, is enclosed in the adhering pericarp (Kiesselbach 1949). The mature kernel consists of pericarp, aleurone, endosperm, embryo, and pedicel.

Development of Endosperm

The endosperm makes up about 85% of the weight of the kernel and is the nutrient source for the germinating embryo. This nutrient source takes the form of intracellular starch grains and protein bodies in different parts of the endosperm (for a review see: Poethig 1982).

The histological description of the development of endosperm has been provided by Randolph (1936), Kiesselbach (1949), and Sass (1976). After double fertilization, the triploid primary endosperm nucleus divides mitotically within 3 to 5 hours, and repeated division continues until a central vacuole in the embryo sac is formed, which is surrounded by a layer of free nuclei embedded in cytoplasm. Cell walls are then formed between the free nuclei, but no

wall is formed next to the central vacuole. This process is repeated until the cavity becomes filled with cellular tissue (Kiesselbach 1949).

The cell divisions occur throughout the endosperm for some time and soon become rare in the inner region. The outermost layer of endosperm then acts as part of the cambium-like meristem and periclinal cell division occurs. The cells close to the outer edge of the endosperm are the last to be formed and are relatively small (Kiesselbach 1949; McClintock 1978). In the central endosperm cells, when mitotic activity decreases at about 10-12 days after pollination (DAP), nuclear size and DNA content per nucleus begin to increase until peak levels are reached at about 14-18 DAP (Kowles and Phillips 1985). Because DNA replication is unaccompanied by cell divisions, the central endosperm cells become highly polyploid (Duncan and Ross 1950; Lin 1977).

In the basal segment of the endosperm, cells are large and funnel from the former central cell of the embryo sac toward the scutellum of the embryo (McClintock 1978). At the base of the endosperm, opposite to the region of placentation, the cells are characterized by cell wall ingrowth (Schel et al. 1984; Shannon et al. 1986) and are modified as conducting cells for transportation of food from the mother plant to the growing endosperm and indirectly to the embryo (Kiesselbach 1949).

At about 10-15 days after pollination, the epidermal

layer starts to differentiate into the aleurone layer (Kyle and Styles 1977). The differentiation of aleurone layer is characterized by the formation of aleurone protein bodies and spherosomes. Spherosomes become closely apposed to the aleurone grains and the plasma membrane during the final stages of maturation (Kyle and Styles 1977). Cell divisions cease at about 20 DAP and the cells complete their differentiation by enlarging and by filling their cytoplasms with starch granules (McClintock 1978).

The endosperm is the nutrient source for the developing embryo. Ultrastructural studies (Schel et al. 1984) show structural changes in the basal endosperm transfer cells (BETC) and in the abaxial and adaxial endosperm cells close to the embryo during early developmental stages of the kernel. The endosperm cells near the basal region of the embryo contains dense cytoplasm and a mass of highly ordered rough endoplasmic reticulum. The BETC region, characterized by wall ingrowth, has an important function in the transport of nutrients supplying the developing embryo (Schel et al. 1984). The ultrastructure of maize endosperm during development has also been reported by Khoo and Wolf (1970) in the study of origin and development of protein granules in maize endosperm, and by Lending and Larkin (1989) in the study of the zein composition of protein bodies during maize endosperm development.

Mutations of Endosperm Altering Quantity and Quality of Starch

Maize endosperm is the major site of starch deposition in the plant during kernel development. About 70-80% of kernel dry weight is starch, and many maize mutants alter quantity or quality of starch in the endosperm. The shrunken-1, shrunken-2, brittle-2, and waxy mutants are examples of maize starch mutants whose corresponding genes have been studied (Chourey 1982).

The shrunken (sh) mutation causes a shrunken or collapsed phenotype of the kernel due to a reduction of nearly 40% starch content as compared to the normal Sh Sh endosperm. Endosperm extracts of sh sh mutants are highly deficient in sucrose synthase activity, and lack a major cytoplasmic protein designated as the Sh protein (Schwartz 1960; Chourey and Nelson 1976, 1979).

The shrunken-2 and brittle-2, two non-allelic mutations, also cause starch deficiency in the maize endosperm. The mutant endosperm contains only 25-30% as much starch as does the normal endosperm, and both mutants are associated with a marked reduction in the level of ADP-glucose pyrophosphorylase (Tsai and Nelson 1966; Dickinson and Preiss 1969).

The waxy mutation affects the quality of starch, but not the quantity, in the endosperm. The starch produced in the normal (Wx) genotype contains 25% amylose and 75% amylopectin, while the starch in homozygous wx mutant is composed of 100% amylopectin (Sprague et al.

1943). The Wx locus, located on chromosome 9, codes for a starch granule-bound enzyme, UDP-glucose starch glucosyltransferase, which transfers glucose from UDP-glucose to starch (Nelson and Rines 1962). Nelson and Tsai (1964) found that in waxy mutants glucose is transfered from adenosine diphosphate glucose to starch at the rate of about one tenth of that in non-waxy maize. The wx mutant retains a residual level of enzymatic activity, which has different biochemical properties than does the Wx protein (Nelson et al. 1978). The Wx protein, a 58 Kd polypeptide, is the monomeric form of the glucosyl transferase enzyme (Shure et al. 1983; Echt and Schwartz 1981).

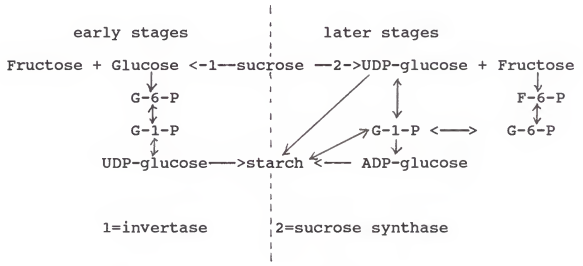
Various types of shrunken endosperm mutants have also been described in barley. Eleven Na-azide induced barley shrunken endosperm mutants expressing xenia (sex) have been reported (Bosnes et al. 1987). The seg2 mutant is a maternal-effect shrunken endosperm mutation in barley. Homozygous seg2 mutants produce shrunken seeds with 15% of normal dry weight regardless of pollen source. The early development of endosperm of the seg2 mutant is arrested due to the delay in endosperm cellularization and antipodal degeneration (Felker et al. 1987).

Starch Formation in Endosperm

In the maize endosperm, sucrose is imported from the photosynthetic tissues and is converted into starch in amyloplasts. After sucrose accumulates in the pedicel

tissue, it moves passively into the pedicel free space, where it is hydrolyzed to glucose and fructose prior to entry into the endosperm via the basal endosperm transfer cells (Shannon 1972; Felker and Shannon 1980; Shannon et al. 1986).

A hypothetical scheme of sucrose-starch conversion during endosperm development was proposed by Tsai et al. (1970) and is shown below:



All endosperm cells except the surface aleurone layer serve for storage of food materials, consisting largely of starch grains. Starch formation begins two weeks or less after fertilization. The first cells to show starch grains are in the upper or crown part of the kernel and the accumulation of starch progresses from upper toward the basal part of the endosperm (Kiesselbach 1949).

Embryo Development

The maize embryo consists of coleoptile, plumule,

scutellum, radicle, and coleorhiza. The mature embryo comprises five or six short internodes and bears a leaf at each node. The first leaf is called scutellum and its outer layer where in contact with the endosperm is specialized to produce enzymes for digesting the starch in the endosperm (Kiesselbach 1949; Abbe and Stein 1954).

Various developmental stages of embryo include proembryo, transition stage, coleoptilar stage and stage 1-6. The first leaf primordia forms at stage 1 and stage 2-6 are reached as the second through the sixth leaf primordia are formed (Sheridan and Thorstenson 1986; Sheridan and Neuffer 1982; Abbe and Stein 1954).

Embryo mutants, which block development at various stages, have been described. For example, dek22 and dek23 are two embryo-lethal maize mutants, which block embryo development prior to the formation of leaf primordia; the mutant embryo remains blocked at the transition stage and undergoes no further development or growth (Clark and Sheridan 1986; Sheridan and Clark 1987).

Sucrose Synthase

Sucrose synthase (UDP-glucose:D-fructose 2-glucosyltransferase, EC 2.4.1.13) is the enzyme catalyzing the reversible reaction UDP + sucrose <---> UDP-glucose + fructose. The role of sucrose synthase in storage tissue is the cleavage of sucrose (Pressey, 1969; Tsai et al.

1970). Although the major role of sucrose synthase is to mobilize sucrose into nucleotide sugars, such as UDP-glucose, in the conversion of sucrose to starch, various forms of nucleoside diphosphate glucoses produced by the cleavage of sucrose can be utilized for the biosynthesis of cell wall polysaccharides (Grimes et al. 1970).

Sucrose Synthase-1 Isozyme

Schwartz (1960) analyzed endosperm proteins in maize mutants that affect starch synthesis and observed that the sh mutation, which is characterized by a shrunken endosperm phenotype, is associated with a complete loss of a major protein designated as the Sh protein. Subsequent analyses by Chourey and Schwartz (1971) and Chourey and Nelson (1976) showed that Sh locus is the structural gene for the Sh protein and the Sh protein is sucrose synthase in the developing endosperm of maize; the starch deficiency of the sh mutant is due to the loss of the Sh-encoded sucrose synthase activity.

Biochemical characterization of the purified sucrose synthase enzyme from maize endosperm was done by Su and Preiss (1978). The enzyme is reported to be a tetramer composed of identical subunits with a molecular weight of 88,000. Sucrose synthase in normal and sh endosperm was genetically analyzed by Chourey (1981a,b) and Chourey et al. (1986). Two sucrose synthase proteins were identified by electrophoric analyses and only the second sucrose

synthase protein was found to be present in the sh-deletion mutant (Chourey 1981a). Sucrose synthase was also detected in maize seedlings by immuno-blot analyses. Sh root extracts show a total of five isozymes due to protomeric interaction of the SS1-SS2 subunits present in the same cell (Chourey et al. 1986).

The Sh gene is on the short arm of chromosome 9 in maize and has been extensively studied at the molecular level. The structure of the Sh gene has been studied by Werr et al. (1985) who found that the sucrose synthase gene is 5.4 kb long, of which 2746 bp are present in the mature mRNA. A cDNA clone of the Sh gene has been isolated (Gupta et al. 1988). The insertion of controlling element Ds at the shrunken locus of maize has been studied by Burr and Burr (1981) and Doring et al. (1981).

Sucrose Synthase 2 Isozyme

Although some sh mutants, including a Sh-deletion strain, show a complete loss of the Sh protein, the mutants retain a low level of sucrose synthase activity ranging about 3 to 5% as compared to normal (Sh) endosperm. This residual enzyme activity is due to the second sucrose synthase isozyme, SS2, which is encoded by a non-allelic gene, Sus (Chourey 1981a; Chourey et al. 1986). Cloning, genetic mapping, and expression studies of the Sus gene have been performed by McCarty et al. (1986) and Gupta et al. (1988).

The SS1 and SS2 enzymes are distinguishable by a difference in electrophoretic mobility in native gels and in the relative abundance of enzyme molecules. SS1 and SS2 have partial antigenic identity and similar enzyme kinetics in the sucrose cleavage reaction. The amino acid composition of SS1 and SS2 are similar, and they share limited structural homologies (Echt and Chourey 1985).

A Sh revertant, Sh-r5, derived upon excision of a Ds element from the sh-m5933 allele (Courage-Tebbe et al. 1983) is the first known Sus null mutation. It has no SS2 protein in any part of the plant including immature embryo, but there is no phenotypic change in the plant. The SS1 protein encoded by the Sh-r5 allele is detected in the immature embryo and endosperm (Chourey et al. 1988). Genomic filter hybridizations with the Sus cDNA clone indicates that the Sus locus in the Sh-r5 strain is not deleted (Chourey et al. 1988).

Tissue Specific Polymerization of SS Subunits

The expression of Sh gene was originally considered to be endosperm specific. However, Springer et al. (1985) detected Sh encoded transcripts in seedlings and Chourey et al.(1986) showed the presence of Sh encoded protein (SS1) in seedlings by immuno-blot analysis.

The SS1 protein is present in endosperm and seedling roots of plants with the Sh genotype; SS2 protein is present in endosperm, embryo and roots of both Sh and sh

genotypes (Chourey et al. 1986). Sucrose synthases from Sh root extracts show a total of five SS isozymes, S1S1S1S1, S1S1S1S2, S1S1S2S2, S1S2S2S2, and S2S2S2S2, due to protomeric interaction of the SS1-SS2 subunits present in the same cell. But endosperm extracts of the same genotype show only SS1 and SS2 homomers. The lack of heteropolymers is attributed to spatial and/or temporal separation of expression of the Sh and Sus genes in endosperm cells (Chourey et al. 1986).

Anaerobic Response in the Maize Plant

The maize seedling responds to anaerobic stress by the synthesis of selective proteins, designated anaerobic proteins (ANP) (Sachs and Freeling 1978). The translation of aerobic messages is repressed and 20 new proteins, including 10 major and 10 minor polypeptides, are produced under anaerobic stress (Sachs et al. 1980). The enzymatic function of four of these anaerobic proteins has been studied: Two alcohol dehydrogenase (ADH) isozymes which are anaerobically inducible in root and mesocotyl (Freeling 1973; Sachs and Freeling 1978), pyruvate decarboxylase (Wignarajah and Greenway 1976), and glucose phosphate isomerase (Kelley and Freeling 1984). All four enzymes are directly involved in glycolysis.

The identification and characterization of cDNA clones to five different mRNA species in the maize root induced

upon anaerobic shock were done by Hake et al. (1985). Upon reintroduction of air, the anaerobic mRNAs disappear rapidly and at approximately the same rate as they are accumulated.

The Shrunken gene in maize also responds to anaerobic stress (Springer et al. 1986; McCarty et al. 1986). Upon anaerobic stress of the young seedling , the level of Shrunken transcripts increases 10 and 20 fold in shoot and root tissue, respectively. However, transcription of the second sucrose synthase gene (Sus) appears irresponsive to anaerobic stress (Springer et al. 1986). The effect of anaerobic stress on expression of sucrose synthase in maize was further examined by McElfresh and Chourey (1988). Following 24 hours of anaerobic treatment, a significant increase in the steady state level of Sh mRNA was detectable; however, there was no detectable increase in sucrose synthase proteins by either native or denaturing Western blot analysis nor was there an increase in sucrose synthase activity.

Immunocytochemical Labeling

Immunocytochemistry is based on the use of labelled antibodies as specific reagents for the in situ localization of specific antigens in tissue (for review see Polak and Van Noorden 1984).

Production of Primary Antibodies

Antibodies are mainly r-globulins and are usually raised by immunizing rabbits, pigs, or mice with antigens for polyclonal antibody production. The Enzyme-Linked ImmunoSorbent Assay (ELISA) against the pure antigen may be used to test the antiserum obtained from these kinds of animals. Another method allowing the production of highly specific antibodies is the monoclonal antibody approach using hybridoma fusion techniques (Davis et al. 1986).

Immunolocalization Methods

Immunofluorescence methods. Coons et al. (1950) were the first to conjugate an antibody with a fluorescent dye and use it to identify an antigen in tissue sections for medical studies. In the early stages the specific antibody was conjugated to fluorescein isothiocyanate (FITC), and the sections were examined using fluorescent microscopy. Subsequently, the method was adapted to become an indirect immunofluorescence technique (Coons et al. 1955) by using a secondary antibody conjugated with FITC.

Immunoenzyme methods. Peroxidase, first introduced by Nakane and Pierce (1966), has lately replaced fluorescent dye as the antibody label in many immunocytochemical reactions. The antibody is visualized by a histochemical reaction for peroxidase based on diaminobenzidine (DAB) reaction.

Gold labeling methods. Antigens in the sections can

also be labelled with colloidal gold particles. These methods were originally introduced for electron microscopy (Faulk and Taylor 1971) as the gold particles are easily visible in the electron microscope. Colloidal gold particles can be attached to protein A from the bacterium Staphylococcus aureus or a secondary antibody for an indirect staining (Romano et al. 1974). The protein A/gold complex binds to the Fc portion of immunoglobulins and acts as the second layer of the immunostain. The secondary antibody conjugated with colloidal gold binds to the primary antibody, thus the antigen is detectable through the colloidal gold particles.

Because the gold complex has an intense red color, the colloidal gold labeling methods are also used for light microscopy. Reduction of the gold particles by silver lactate to give an intense black color greatly increases the sensitivity of the immunogold methods (Holgate et al. 1983). This silver enhancement method is useful for detection of antigens in paraffin sections.

Examples of Immunolocalization in Plants

Immunolocalization has been used extensively in medical studies to localize antigens of specific diseases. Recently, immunocytochemical techniques have been used to localize several proteins in plants as well, and the immunogold method is widely used in these studies.

Immunogold staining was used to localize the storage

protein zein in protein bodies isolated from maize endosperm. The results indicated a spatial/temporal difference in zein synthesis (Lending et al. 1988). Immunogold labeling was also used to localize chalcone synthase, the key enzyme of flavonoid pathway, to ribosome-bearing endoplasmic reticulum membrane in the epidermis of buck wheat hypocotyls (Hrazdina et al. 1987). The L3 protein of maize was localized to the surface phospholipid monolayer of lipid bodies by immunocytochemical electron microscopy (Fernandez et al. 1988). Glutamine synthase was localized in leaves and cotyledons of young tomato plants using the immunogold technique (Botella et al. 1988). The vacuolar H^+-ATPase of maize root tip cells has been localized at the EM level using rabbit polyclonal antibodies and protein A-colloidal gold. The results show that H^+-ATPase is mainly located on the tonoplast and Golgi membranes (Hurley and Taiz 1989).

MATERIALS AND METHODS

Maize Strains

Kernel

The W22 inbred line (Sh Sh) was used as the wild type or non-shrunken line; sh bzm4, recovered by McClintock, was used as the Sh-deletion shrunken mutant. The Sh-r5 revertant of a shrunken (sh) mutant was also used, due to its lack of SS2 protein.

Root

The wild-type or non-shrunken lines were Pioneer inbreds designated Pio3055 and Pio3165. The Sh-deletion mutant was sh bzm4. Seedlings for anaerobic treatment were submerged in 5-10 mM TRIS-HCl (pH 7.5) for 24 hours.

Immunolocalization - Light Microscopy

Tissue Preparation for Paraffin Sections

Kernel. Maize plants were grown in the green house and were harvested at 8, 9, 10, 12, 14, 16, and 20 days after pollination (DAP). To collect samples, the ear bag was lifted, and the husks were slit and carefully peeled back. Individual kernels were removed with a surgical

blade and forceps. Collected kernels were put in scintillation vials with water and immediately brought to the laboratory. Before fixation, the maternal tissue was removed and the kernel was trimmed on both sides parallel to the embryonic axis as described by Sass (1945).

Kernels were fixed in a formalin acetic alcohol (FAA) solution shown in the Appendix. The vials containing kernels and fixative were placed under vacuum until no bubbles appeared. The FAA was then changed. Kernels were in fixative for at least 24 hours before processing further.

Kernels were dehydrated through a tertiary butyl alcohol series shown in the Appendix. Infiltration was done in a 60°C oven. A piece of filter paper was put in the vial above the kernels, and paraffin chips were placed on it so they did not touch kernels directly. Ten paraffin chips were added to each vial every 2 hours until there was enough paraffin to cover the samples. Vials were left in the oven for 2 days before lids were removed. A day later kernels were aligned in peel-away embedding molds and cast into paraffin blocks.

The blocks were removed from the molds, trimmed, and mounted on wooden blocks for sectioning. In order to prevent crumbling in sections, the cutting surfaces of older kernels (greater than 16 DAP) were soaked in distilled water for several days to soften the starch abundant endosperm (Larkin et al. 1952). Paraffin ribbons

were cut with a Shandon microtome at 10 μm thickness and were inspected under a dissecting microscope to select the desired sections. Glass slides were coated with Haupt's adhesive and flooded with 3% formalin before sections were mounted. Slides with sections were dried over night on a warm tray at 40°C.

Root. Roots from 5-7 day-old control and anaerobically (AN) stressed seedlings were cut at the tip to a length less than 1 cm, fixed in FAA, dehydrated through a tertiary butyl alcohol (TBA) series, and cast into paraffin blocks. The method was the same as described for the kernels, except that roots were left in TBA solutions for half the time given for kernels.

Immunolocalization for Light Microscopy

Preparation of mono-specific SS1 and SS2 antisera has been described previously (Echt and Chourey 1985). The cellular localization of SS isozymes was done using the immunogold silver staining (IGSS) kit (Janssen Life Products). The following protocol available from Janssen Life Science Company was used for the IGSS reactions.

Slides with 10 μm sections were deparaffinized in xylene for 10 minutes, rehydrated with gradient ethanol: 100%-- 95%--85%--70%--30%--water, 5 minutes for each step, and then rinsed with 0.5% BSA-Tris plus 20 mM NaN3 solution. The preparations around the sample area were dried and covered with 50-200 ul of 5% heat-inactivated

normal goat serum in 0.1% BSA-Tris. After 20 minutes, normal serum was replaced with an equal amount of 1:500 diluted SS1 or 1:200 diluted SS2 antiserum for kernel sections and 1:200 diluted SS1 and SS2 antiserum for root sections. The concentrations used were determined to be the highest dilutions which still resulted in a strong positive signal for the respective proteins; thus, cross-reaction of SS1 antiserum with SS2 protein and vice versa was minimized. Slides were incubated overnight in a humidified petri dish. After three 10 min washes with 0.1%BSA-Tris plus 20 mM NaN_3 solution, slides were incubated with 1:100 diluted secondary antibody comprised of goat anti-rabbit IgG linked to colloidal gold (Auro Probe LM) for 30 min in a humid environment.

Subsequently, slides were washed two times for 5 min each with Phosphate-buffered saline (PBS),and post-fixed with 2% glutaraldehyde for 15 min. After two washes with PBS and three washes with water for 3 min each, and a wash with 0.2M citrate buffer for 2 min, slides were incubated for 7 min with freshly prepared silver enhancement reagents. The slides were washed with excess distilled water, dehydrated through gradient ethanol, stained with Fast Green in 95% ethanol, and permanently mounted for microscopic evaluation. The positive reaction of IGSS reagents is identified as dark brown to deep black stain in the tissue sections.

The immunolocalization method for root section is

slightly different in the steps after primary antibody treatment. Briefly, after three 10 minute washes with PBS solution, slides were treated with 1:40 diluted secondary antibody comprised of goat anti-rabbit IgG linked to colloidal gold (Auro Probe LM) and incubated for 60 minutes in a humid environment.

Subsequently, slides were washed 3 times for 5 min each with PBS buffer and 3 times for 3 min each with distilled water, and incubated for 15 min with silver enhancement reagent (Janssen Life Products) by mixing an identical number of drops of enhancer solution and initiator solution. Then the slides were washed with excess distilled water, counterstained with Fast Green, and permanently mounted.

Microscopy

Permanently mounted sections were examined using a Nikon Optiphot light microscope. Photomicrographs were taken with Kodak Panatomic-X 32 black and white film.

Immunolocalization - Electron Microscopy

Scanning Electron Microscopy

The 14 DAP kernels of Sh and sh genotypes were fixed in FAA solution as described in "tissue preparation for paraffin sections." The kernels were cut longitudinally in the center, dehydrated through ethanol series up to 100%

ethanol, and then dehydrated by the critical point drying method (Klomparens et al. 1986). The dry kernels were coated with gold by sputter coating, glued on the stubs, and examined under a Hitachi-450 scanning electron microscope.

Preparation of Tissue Sections for Transmission Electron Microscopy

Kernel. Maize kernels at 12 and 16 DAP were cut into 1 mm thick slices along embryo axis, fixed at 4°C overnight in cacodylate buffer (pH 7.2) containing 3% glutaraldehyde, 2% paraformaldehyde, 2.5% dimethyl sulfoxide, and 0.5% acrolein. The tissue was then washed three times for 15 min each with 0.1M cacodylate buffer and post-fixed in OsO_4 solution at 4°C overnight. After three washes with water for 15 min each, specimens were dehydrated in a graded ethanol series and embedded in LR White. Polymerization of LR White was done in a 60°C oven for 18-24 hr. The steps of dehydration and infiltration were as follows:

Steps	solution	time needed
1	25% EtOH	15 min
2	50% EtOH	15 min
3	75% EtOH	15 min
4	95% EtOH	15 min twice
5	3 parts EtOH : 1 part LR White	overnight
6	1 part EtOH : 1 part LR White	12 hr
7	1 part EtOH : 3 parts LR White	overnight
8	LR White	overnight

After kernels were embedded, tissue blocks were trimmed to less than 0.5 x 0.75 mm. Sections were cut with a LKB ultramicrotome and silver sections were picked up with 100 mesh formvar coated nickel or copper grids. Nickel grids were used specifically for immunostaining. When tissue was prepared for immunostaining , the step of post fixation with OsO_4 was deleted.

Root. Roots of control and AN seedlings were cut into 1mm segments and fixed for 2 hr in cacodylate buffer containing 1% glutaraldehyde and 4% paraformaldehyde for immunostaining tissue and in buffer containing 2% glutarldehyde and 2% paraformaldehyde for non-immunostaining tissue. The other steps were the same as for the kernels.

Immunolocalization for Electron Microscopy

Protein A method. The ultrathin sections mounted on nickel grids were washed with water two times for 10 min each, incubated with 1% ovalbumin for 10 min, and then transferred to 1:200 diluted SS1 or SS2 antibody for 1 hr. After two 10 min washes with PBS solution, grids were incubated with 1:20 diluted protein A complex solution for 30 min. Subsequently, grids were washed with PBS buffer two times for 10 min each and with water for 5 min, then post stained with 5% Uranyl Acetate and Lead Citrate.

Secondary antibody method. The ultrathin sections mounted on 100 mesh nickel grids were washed with water for 10 min, then incubated with 5% normal serum for 15 min to block non-specific binding. Grids were then incubated with diluted SS1 or SS2 antibody for 1 hr, washed with BSA buffer two times for 5 min each, and incubated with 1:100 diluted AuroProbe EM reagent (Janssen products) for 1 hr. After two 5 min washes with BSA buffer and PBS buffer, the grids were postfixed with 2% glutaraldehyde in PBS for 15 min, washed with distilled water 2 times for 5 min each, and contrasted using 5% Uranyl Acetate and Lead Citrate.

Silver enhancement. After postfixation with glutaraldehyde in PBS buffer and washing with water, the sections were washed three times 5 min in PBS, three times 3 minutes in distilled water, then incubated with silver enhancement reagent for 7 minutes and washed with excess distilled water two times 5 minutes.

RESULTS

Localization of Sucrose Synthases in Maize Kernels

Localization of Sucrose Synthases in Kernels of Sh and sh Genotypes

Serial sections of immature kernels of different genotypes at various stages of development, from 8 DAP to 16 DAP, were examined for the accumulation of SS proteins. Immunolocalization was done using either SS1 or SS2 antibody followed by a secondary antibody conjugated to colloidal gold particles and then by a silver enhancement reagent. The positive reaction of immuno-gold silver stain (IGSS) resulted in a dark brown to deep black stain. Preimmune serum was used to identify non-specific stain on the sections. The sections of 12 DAP Sh kernels treated with the same dilution of preimmune serum and SS1 antiserum are shown in Figure 1 A-B. The minimal level of IGSS with preimmune serum (Fig. 1A) and much stronger signal with SS1 antiserum (Fig. 1B) indicated that the increased signal in the latter was specifically due to reaction of SS protein.

The SS1 and SS2 proteins are immunologically similar but not identical (Echt and Chourey 1985); SS1 antibody will cross-react with SS2 protein and vice versa. However,

Western blot analyses show stronger immunosignals in homologous antigen-antibody reactions (i.e. SS1 antigen with SS1 antibody) than in heterologous reactions. Various levels of dilutions of SS1 or SS2 antibody were tested to find the highest dilution to give a strong homologous reaction and weak heterologous reaction. When sections of Sh and sh kernels were treated with 1:500 diluted SS1 antiserum, no detectable signal was seen in sh endosperm (Fig. 1C) although a strong signal was seen in Sh endosperm (Fig.1B). Because nearly 95% of the SS protein in Sh endosperm is due to the Sh-encoded SS1 protein (Chourey 1981a) and because there was no reaction with SS1 antiserum in sh endosperm, the IGSS signal in Sh endosperm was concluded to be due to reaction with SS1 protein. The SS2 protein in sh endosperm was detectable when sections were treated with 1:200 diluted SS2 antibody (Fig. 1D).

SS1 Protein in Developing Sh Endosperm. The distribution of SS1 protein in Sh kernels at various developmental stages is shown in Figure 2. The SS proteins were barely detectable in the kernel at 8 DAP, only part of the aleurone layer showed a positive signal (Fig. 2A). During the development of the kernel, SS1 protein in the endosperm was first seen in the crown region directly above the embryo (Fig. 2B), then it was distributed from crown region toward the central part (Fig. 2C-E). A close-up view of the gradient of Sh expression in the endosperm at 9 DAP indicated that the SS1 protein level was higher in the

crown region on the embryo side (Fig. 3A) than in the crown region away from embryo side, and no positive signal for SS1 protein was detected at the base of the endosperm designated as basal endosperm transfer cell (BETC) (Fig. 3C). This gradient of SS1 protein was seen in developing endosperm up to 12 DAP (Fig. 2B-D). In 14-16 DAP endosperm, the level of SS1 protein increased further and the SS1 protein was distributed throughout the crown region and central part of the endosperm but not in the BETC region (Fig. 2E-F).

The first positive signal for SS protein in the aleurone layer was seen just above the embryo (Fig.2A). The entire aleurone layer showed positive SS signal by the 10 DAP stage (Fig. 2C). At the 16 DAP stage, the IGSS signal in the aleurone layer was reduced at the region which first showed the IGSS signal at the earlier stage of development. A sharp discontinuation of the immuno-signal in the aleurone layer was seen at the point where these cells develop into the BETC region.

The earliest developmental stage of the embryo showing a positive IGSS signal was 9 DAP (Fig.2B). After 10 DAP a higher level of SS protein was detected in the vascular tissue, shoot apex, and radicle of the embryo (Fig.2C-F). At 16 DAP a significant level of differentiation of the embryo was correlated with a high level of tissue and cellular specificity of SS protein (Fig. 2F). A much lower level of SS1 protein was in the scutellar region than in

coleoptile, primodial leaves, radicle and coleorhiza; the radicle and coleorhiza showed the highest level of SS1 protein.

SS2 protein in developing sh kernels. The Sh-deletion shrunken mutant, sh bz-m4, was used to specifically localize the SS2 protein in the kernel. Kernels of various developmental stages were examined for expression of the Sus gene (Fig.4). In the 8 DAP endosperm, only embryo and endosperm cells near the base of the embryo showed IGSS signal; the strong staining was visible in the parenchyma and vascular tissue of the pedicel (Fig. 4A). The SS2 protein was detectable at the crown, central, and basal region of the endosperm after the 10 DAP stage (Fig. 4B-C). The distribution of SS2 protein in different parts of the 10 DAP endosperm is shown in Fig. 5. A higher level of SS2 protein was seen in the outer layers of the crown region (Fig. 5 A,C) than in the mid-crown region (Fig.5B), and the SS2 protein was also detected in the BETC region (Fig.5D). The mid-crown region showed a very low density of IGSS signal at 12 DAP endosperm (Fig. 4C). By 14 DAP two cavities had developed in the sh kernel and by 16 DAP one large cavity had formed. The gradient of SS2 accumulation in the sh endosperm was otherwise similar to the wild-type genotype, except that the density of signal was markedly weak. The pattern of SS distribution in the developing embryo of the sh genotype was the same as it was in the embryo of the Sh genotype.

Fig. 1 A-D. Localization of SS proteins at upper crown region in 12 DAP kernels of Sh (A-B) and sh (C-D) genotypes. Photographs taken from the region shown in the hatched square. A,B- Sh kernels stained with preimmune serum (A) and SS1 antibody (B). C,D- sh kernels stained with SS1 antibody (C) and SS2 antibody (D).
Original magnification x 31.25

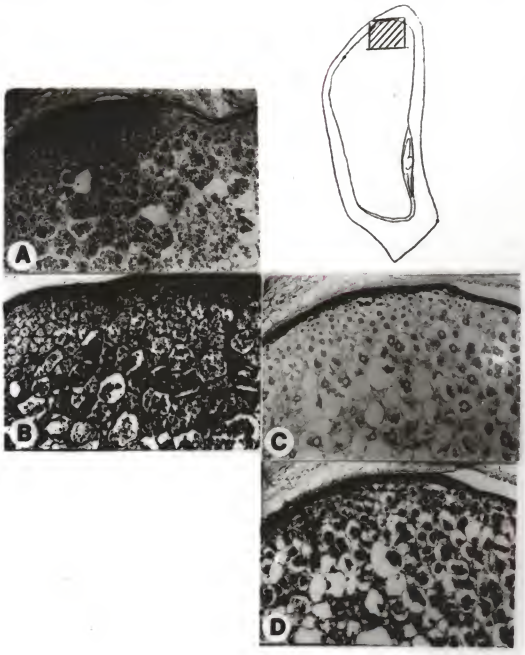

A
B
C
D

Fig. 2 A-F Localization of SS proteins in Sh kernels at different developmental stages. Longitudinal sections of paraffin embedded Sh kernels stained with 1:500 diluted SS1 antiserum at the developing stages indicated: A- 8 DAP. B- 9 DAP. C- 10 DAP. D- 12 DAP. E- 14 DAP. F- 16 DAP. Original magnification x 6.25.

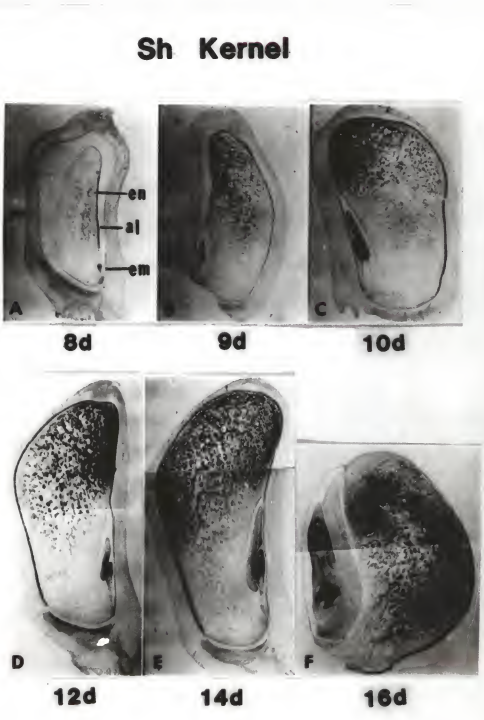

Sh Kernel
en
al
em
A
B
C
8d
9d
10d
D
E
F
12d
14d
16d

Fig. 3 A-C. Expression of SS proteins in different parts of Sh kernel at 9 DAP stage. A- Crown region above embryo. B- Crown region opposite to embryo side. C- Basal endosperm transfer cell. Original magnification x50.

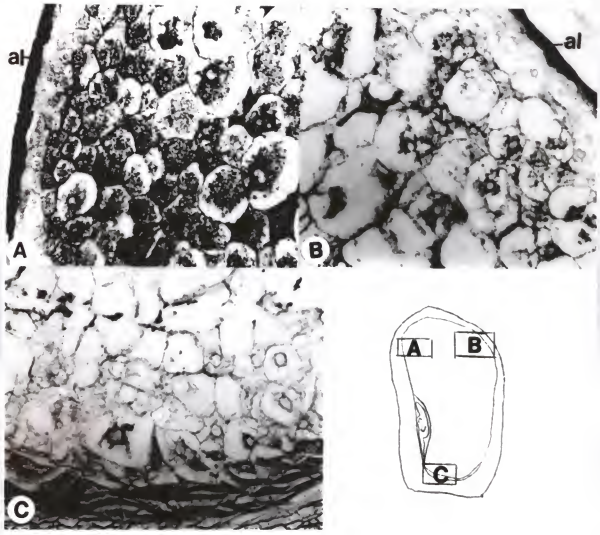

al
al
A
B
C
A
B
C

Fig. 4 A-E. Localization of SS2 proteins in sh bz-m4 kernels at different developmental stages. Longitudinal sections of paraffin embedded sh kernels stained with 1:200 diluted SS2 antiserum at the developing stages as indicated: A- 8 DAP. B- 10 DAP. C- 12 DAP. D- 14 DAP. E- 16 DAP. Original magnification x 6.25.

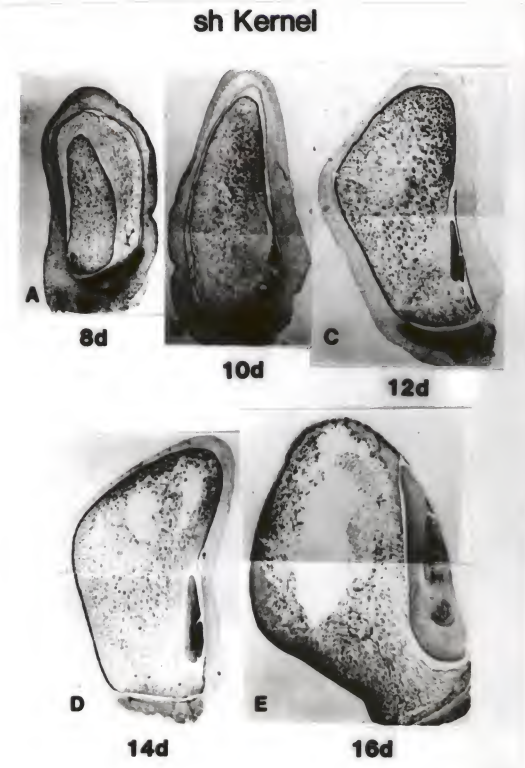
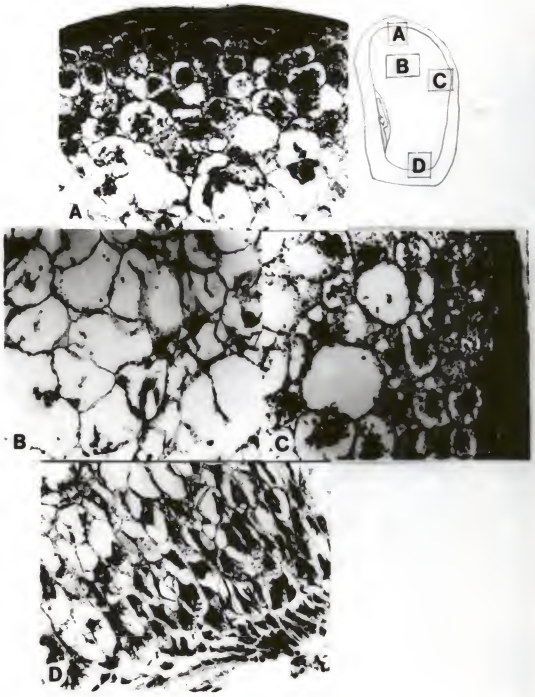

sh Kernel
A
8d
10d
C
12d
D
14d
E
16d

Fig. 5 A-D. Expression of SS2 protein in different parts of sh bz-m4 kernel at 10 DAP stage. A- Upper crown region. B- Mid-crown region. C- Crown region opposite to embryo side. D- Basal endosperm transfer cell. Original magnification A,B,D- x50. C- x100.


A
B
C
D
A
B
C
D

Spatial/Temporal Localization of SS1 and SS2 Isozymes in Relation to Starch Accumulation

Although several developmental stages of the kernel were examined for localization of the SS protein, 12 and 16 DAP stages were of particular interest, as the Sh locus is known to undergo a major increase in expression during this specific phase of development (Chourey 1981a).

Figures 6 and 7 show the cellular localizations of SS proteins and starch in immature kernel at 12 and 16 DAP stages in the Sh and sh genotypes. The lack of IGSS with a pre-immune serum and a strong signal with the SS1 antibody indicated that the positive signal was specifically due to the SS protein. In the Sh kernel, the SS1 protein at 12 DAP stage was much higher in the crown region than in the central part of the endosperm, and it was barely detected at the BETC region (Fig. 6B). At 16 DAP the spatial pattern remained nearly the same as at the 12 DAP stage of development except that the SS1 protein was detectable in a much larger portion of the 16 DAP endosperm. A significant increase in the density of signal was seen at the 16 DAP stage as compared to the earlier stage, eg. 12 DAP (Fig. 6B and Fig. 7A).

The kernel sections were also stained with I-KI solution to detect starch accumulation in the endosperm. Starch accumulation in the 12 DAP Sh endosperm was detected mostly in the crown region (Fig. 6C). At 16 DAP the deposition of starch was in a much larger portion of the endosperm (Fig. 7B). However, no starch accumulation was

seen in the BETC region. The staining pattern of starch at 12 and 16 DAP coincided with Sh expression (Figs.6 B-C and 7 A-B).

Similar analyses were done on the Sh-deletion strain. When sh kernels were treated with SS1 antiserum using the same level of dilution as was used for wild type kernels, no detectable signal was seen in the endosperm (Fig.6 B and D). The aleurone layer, however, showed a strong positive signal (Fig. 6D). In the sh kernels at 12 DAP, the SS2 protein was detected at the crown, central, and basal regions of the endosperm (Fig. 6E). At 16 DAP, cells in the central region of the sh endosperm were lost (Fig. 7 C-D).

The starch accumulation in the sh kernel was similar to that in the Sh kernel, but there were fewer starch grains in the 12 DAP endosperm (Fig. 6F) and the cells of the central crown region were lost by 16 DAP (Fig. 7D). The starch accumulation did not coincide with the distribution of SS2 protein in sh endosperm. For example, no starch accumulation was seen in the BETC region and aleurone layer, but SS2 protein was detected in these two regions (Fig. 6 E and F).

Figure 8 shows close-up photographs of cellular

Fig. 6 A-F. Localization of SS proteins and starch in 12 DAP kernels of Sh and sh genotypes. Longitudinal section of paraffin -embedded Sh (A-C) and sh (D-F) kernels at 12 DAP stained with Fast Green plus as indicated: A- pre-immune serum. B -SS1 antiserum. C -I-KI. D -SS1 antiserum. E- SS2 antiserum and F -I-KI. Abbreviations are: al-aleurone; en-endosperm; em-embryo; pe-pericarp. Original magnificaiton x7.

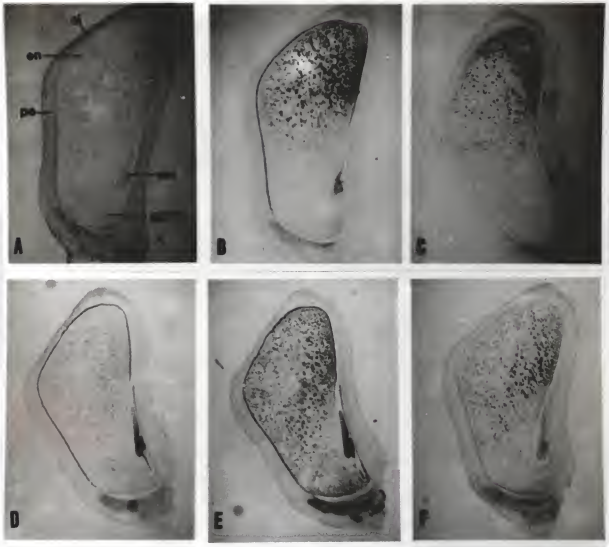

en
pa
A
B
C
D
E
F

Fig. 7 A-D. Localization of SS proteins and starch in 16 DAP kernels of Sh and sh genotypes. Longitudinal section of paraffin-embedded Sh (A-B) and sh (C-D) kernels at 16 DAP stained with Fast Green plus as indicated: A- SS1 antiserum. B- I-KI. C- SS2 antiserum and D- I-KI. Abbreviations are: al-aleurone; co-coleoptile; pl- plumule; sc- scutellum; ra-radicle; cr-coleorhiza. Original magnification x7.

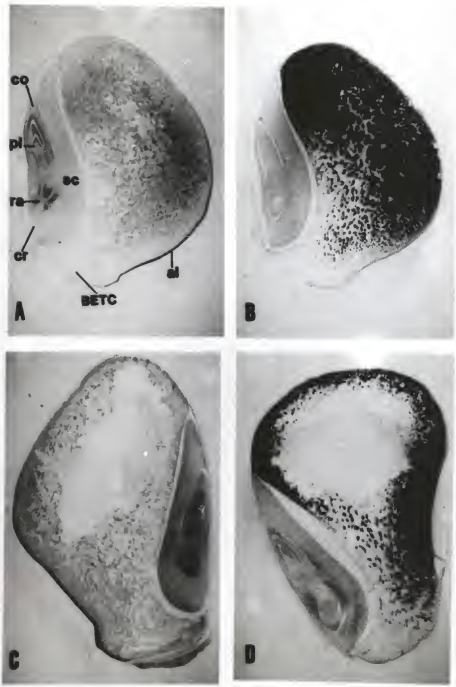

co
pl
ra
cr
bc
BETC
al
A
B
C
D

Fig. 8 A-C. Localization of SS proteins in basal region of the kernel. A close-up view of the longitudinal section of the kernel showing the basal region at the 12 DAP Sh (A and B) and sh (C) genotypes stained with Fast Green plus as indicated: A- SS1 antiserum. B and C- SS2 antiserum. Original magnification x18 (A) and x20 (B and C).

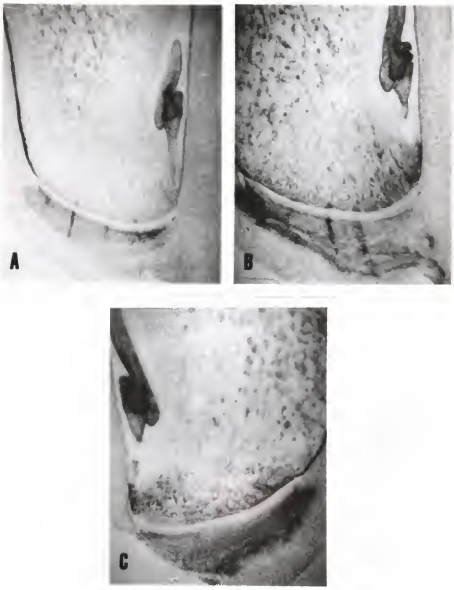
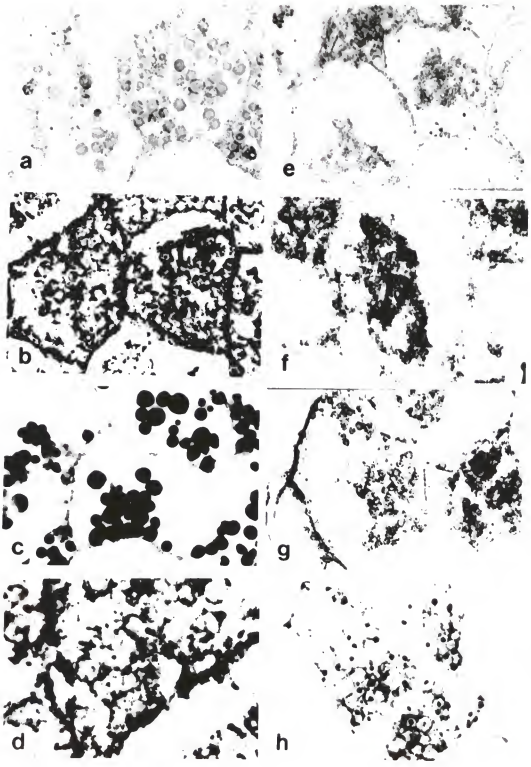

A
B
C

Fig. 9 a-h. Expression of SS protein in cells at crown region in Sh (a-d) and sh (e-h) kernels. 12 DAP Sh kernel stained as indicated: a- preimmune serum. b- SS1 antiserum. c- I-KI. d- SS1 antiserum, oil immersion photo of b. e, f- 12 DAP sh kernel stained with SS1 antiserum (e) and SS2 antiserum (f). g- 14 DAP kernel stained with SS2 antiserum. h- oil immersion photo of g.
Original magnification a,b,c,e,f,g- x156.25. d,h- 312.5


a
e
b
f
c
g
d
h

localization of the SS protein in the BETC region of the Sh and sh genotypes at 12 DAP stage. An important feature of the sh genotype was that the BETC region gave a positive signal with the SS2 antibody (Fig. 8C). An examination of the Sh genotype showed that the BETC region, which gave no detectable signal with the SS1 antibody (Fig. 8A), reacted with the SS2 antibody (Fig. 8B). In addition, the BETC region showed no major quantitative difference in intensity of IGSS signal with the SS2 antibody between the Sh and sh genotypes (Fig. 8 B-C).

A higher magnification view of SS protein accumulation in cells of Sh and sh endosperm is shown in Fig. 9. The Sh kernel sections stained with preimmune serum showed an undetectable IGSS signal (Fig.9a) but showed a strong signal when stained with SS1 antiserum. The accumulation of SS1 protein was seen in the cytoplasm around starch grains and the cell wall (Fig. 9 b and d). In sh endosperm, the accumulation of SS2 protein was also seen around the starch grain in the cytoplasm but not close to the cell wall (Fig. 9 f and h).

Cavity Formation in sh Endosperm

In the sh endosperm, the cells in the crown region showed very low levels of IGS staining at 12 DAP (Fig. 4C), and those cells were broken down by 14 DAP (Fig. 4 D and E). Two cavities were seen in the crown region at 14 DAP. Only a column of cells connected the crown cells with the

basal cells, the cells on either side of this column having been lost (Fig. 4D). At 16 DAP, cells in the central region of the sh endosperm were lost, resulting in one large cavity (Fig. 4E).

Kernels of Sh and sh genotypes at 14 DAP were also examined using scanning electron microscopy (SEM) to observe the cavity formation. Two cavities of disimilar size in the crown region were seen in the sh kernel (Fig. 10B) but not in the Sh kernel (Fig. 10A). As seen in the higher magnification, starch grains in the Sh genotype (Fig. 10C) were larger and greater in quantity per cell than that in the sh genotype (Fig. 10D). In the sh endosperm, the cell walls, which would break down eventually, did not show any difference as compared to the cell walls of the same region in the Sh endosperm (Fig. 10C and D). Differences in cell wall thickness or structure in this part of the endosperm were not readily detectable.

Semithin and ultrathin sections of the crown region of Sh and sh endosperm at 12 DAP were examined to see if there was a difference in cell walls at this stage. Cell wall breakdown (Fig. 11B) and discontinuation in the cell wall (Fig. 11D) were observed in the crown region of the sh endosperm using light and electron microscopy, respectively; whereas no such breakage of cell walls was seen in Sh endosperm (Fig. 11A and C).

Fig. 10 A-D. Scanning electron micrographs of 14 DAP endosperm of Sh (A,C) and sh (B,D) genotypes. A- crown region of Sh 14 DAP kernel. B- Two cavities in the crown region of sh 14 DAP kernel. C- higher magnification of panel A, showing cell wall and starch grains. D- higher magnification of panel B at the area where cells are going to break down.

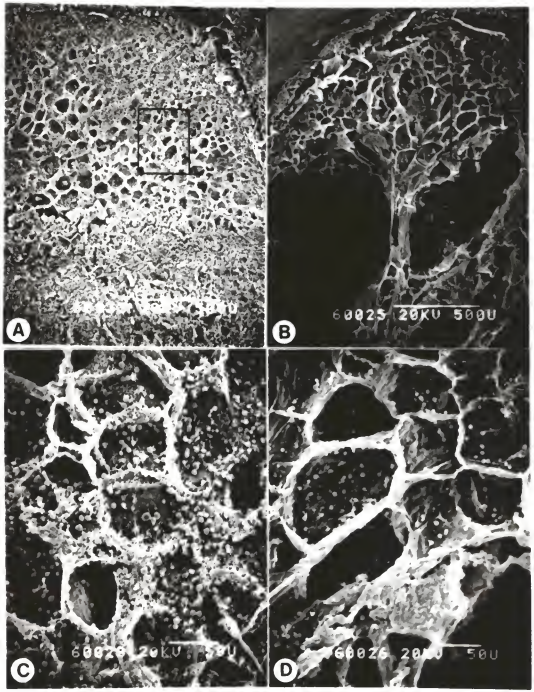
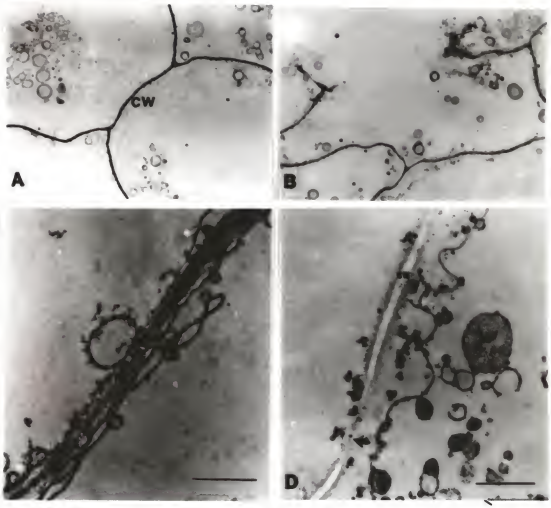

A
B
60025 20KV 500U
C
D

Fig. 11 A-D. Endosperm cell walls in Sh (A,C) and sh (B,D) gehotypes at 12 DAP. A,B- Semi-thin sections of crown region in Sh (A) and sh (B) endosperm. C,D- Electron micrographs showing cell walls of crown region cells in Sh (C) and sh (D) endosperm. The arrow shows the discontinuation of cell wall in sh endosperm.
Original magnification A,B- x 312.5
CW: cell wall. Pb: protein body. Bar= 1μ

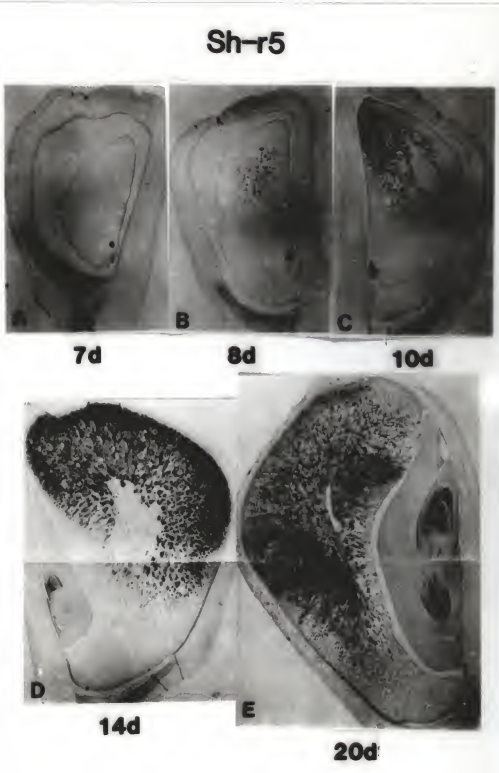

CW
A
B
D

Localization of SS1 Protein in Sh-r5 kernel

The Sh-r5 genotype, characterized by the Sus-null mutant, was used to specifically localize SS1 protein in the kernel. At the 7 DAP stage, there was no detectable SS1 protein in the endosperm (Fig. 12A). The SS1 protein began accumulation in the crown region of endosperm at 8 DAP (Fig. 12B). At 10 DAP SS1 protein was detected at the crown region particularly on the adaxial side of endosperm and there was a small cavity in the crown region (Fig. 12C). After the 14 DAP stage, more SS1 protein was present in the crown region, but still a cavity was present in the mid-crown region (Fig. 12D). The cavity in the mid-crown region became smaller at the 20 DAP stage, and SS1 protein was localized in this region (Fig. 12E).

The pattern of SS1 protein distribution in the Sh-r5 endosperm is similar to that in the Sh endosperm, i.e. SS1 protein was in the crown and central region but not in the BETC region. The difference in the reaction with SS1 antibody between Sh and Sh-r5 endosperm was on the aleurone layer. The aleurone layer in the Sh-r5 kernel did not show IGS staining until 20 DAP, at which stage a small portion of the aleurone layer near the bottom of the endosperm showed IGS stain (Fig. 12E). The SS1 protein in the embryo of the Sh-r5 kernel was not detectable until the 14 DAP stage (Fig. 12A-D), while in the embryo of the Sh kernel the SS protein was detected as early as at the 9 DAP stage.

Fig. 12 A-E. Localization of SS proteins in Sh-r5 kernels at different developmental stages. Longitudinal sections of paraffin embedded Sh-r5 kernels stained with 1:500 diluted SS1 antiserum at the developing stage as indicated: A- 7 DAP. B- 8 DAP. C- 10 DAP. D- 14 DAP. E- 20 DAP. Original magnification x 6.25.


Sh-r5
A
B
C
7d
8d
10d
D
E
14d
20d

The pattern of SS accumulation in the embryo of Sh-r5 was similar to that of wild-type Sh and sh genotypes.

Immunolocalization of SS Protein Using Electron Microscopic Cytochemistry Techniques

The ultrastructure of 15 DAP maize endosperm cells are shown in Fig. 13. The aleurone layer contained a large number of protein bodies and spherosomes, and no starch grains were observed in this layer (Fig. 13A). Starch grains and protein bodies were the predominant components in cells of developing endosperm, and the size of starch grains in the endosperm cells increased with distance from the aleurone layer (Fig. 13B).

The protein encoded by the Wx locus is a well known starch granule bound protein, therefore, Wx antiserum as well as preimmune sera were used as controls to verify the immunolocalization techniques (The Wx antiserum was a gift from Dr. Nina Fedoroff, Carnegie Institute, Baltimore, MD). Immunostaining with a concentration of preimmune serum similar to that used for the primary antiserum gave very low levels of nonspecific staining (Fig. 14A). In the sections treated with Wx antibody, colloidal gold marker was predominantly limited to the starch grains; background labeling over cytoplasm, organelles, and cell walls was extremely low (Fig. 14B).

Immunostaining Sh endosperm cells at the crown region with SS1 or SS2 antiserum revealed that both SS1 and SS2 proteins were present in the cytoplasm but not on the cell

Fig. 13 A-B. Electron micrographs of Sh kernel at 15 DAP. A- aleurone and subaleurone at crown region. Bar= 1μ. B- cells of crown region. al: aleurone layer. PB: protein body. MT: mitochodria. sg: starch grain. Bar= 2μ

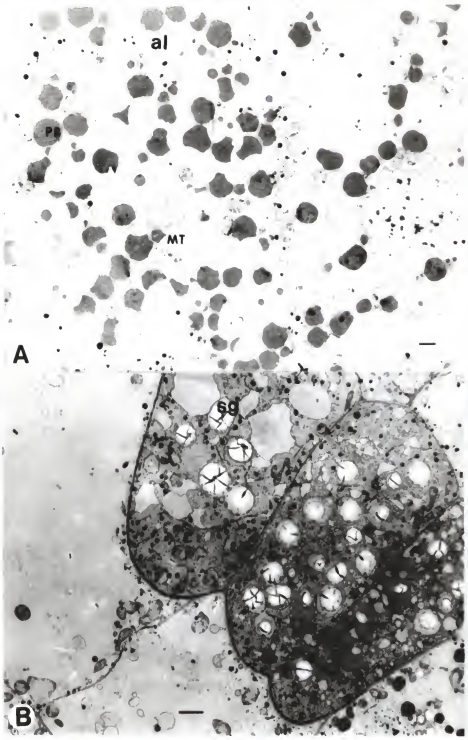

al
PB
MT
A
sg
B

Fig. 14 A-B. Electron micrographs showing immunolocalization of Wx protein in Sh endosperm at 15 DAP. Ultrathin sections of 15 DAP Sh endosperm stained with preimmune serum (A) and Wx antibody (B), then stained with secondary antibody, uranyl acetate, and lead citrate. sg: starch grain. ER: endoplasmic reticulum. CW: cell wall Bar=1μ

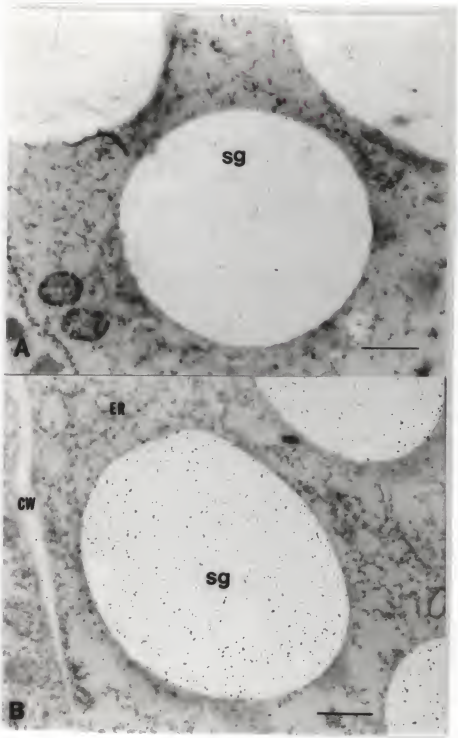

sg
A
ER
CW
sg
B

Fig. 15 A-B. Electron micrographs showing localization of SS proteins in endosperm cells at 15 DAP. A- sections stained with SS1 antiserum. B- sections stained with SS2 antiserum. sg: starch grain. Bar= 1μ

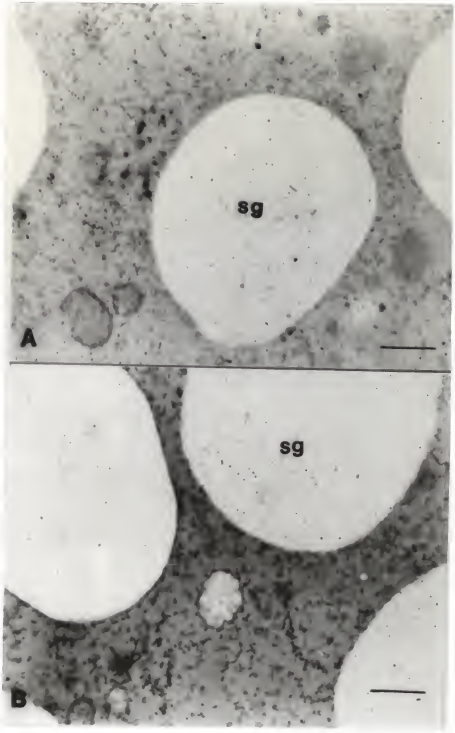

sg
A
sg
B

Fig. 16 A-B. Electron micrographs showing localization of Wx protein in 15 DAP Sh endosperm using silver enhancement reagent. After stained with Wx antibody and secondary antibody, sections were treated with silver enhancement reagnet. A- sections stained with preimmune serum. B- sections stained with Wx antibody. sg: starch grain. Bar=2μ

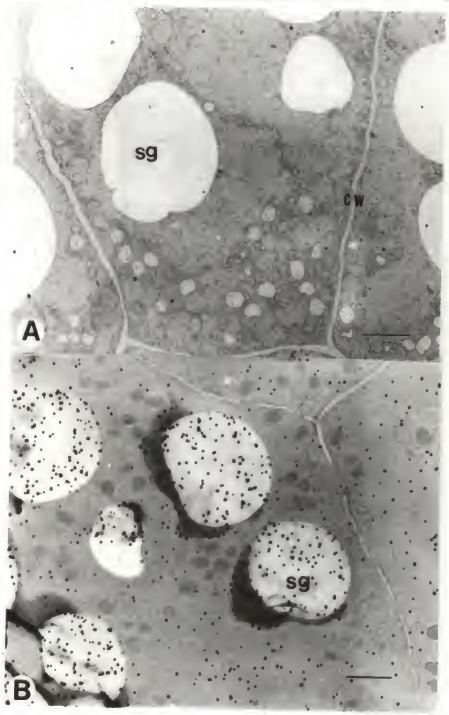
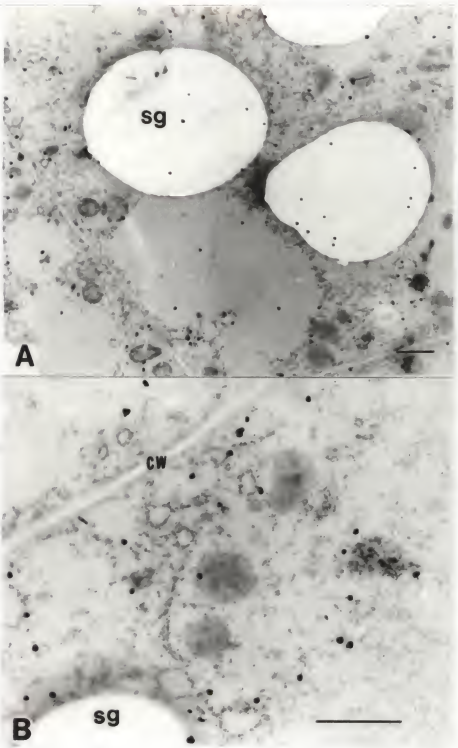

sg
cw
A
sg
B

Fig. 17 Electron micrographs showing localization of SS1 proteins in 15 DAP Sh endosperm using SS1 antiserum and silver enhancement reagent. A- lower magnification. B- higher magnification. sg: starch grain. cw: cell wall. Bar=1μ


sg
A
CW
B
sg

walls or other organelles; the colloidal gold particles were dispersed over the starch grains and cytoplasm (Fig.15 A and B). A silver enhancement reaction was used to amplify the size of gold particles, so that the signal of SS protein could be seen under a lower magnification field of TEM and the distribution of SS protein within a cell could be better observed. The results of silver enhancement on immunostaining with Wx antiserum and SS1 antiserum are shown in Fig. 16 and 17. Colloid gold marker was primarily localized on starch grains of sections stained with Wx antiserum (Fig. 16B), but was dispersed in the cytoplasm of sections stained with SS1 antiserum (Fig. 17 A and B). Thus, no specific localization of SS protein to the cell wall, starch grains, or other organelles was observed in the endosperm cells.

Localization of Sucrose Synthases in Root of Maize Seedlings

Immunolocalization of SS Proteins in Sh and sh Roots Under Aerobic and Anaerobic Conditions

Cross and longitudinal sections of control and AN primary roots were used to localize SS proteins at the cellular level (Fig. 18-21).

Figure 18 shows the immunolocalization of SS proteins, using SS1 antiserum, in serial root cross and longitudinal sections of the Sh genotype. An AN longitudinal section treated with preimmune serum and control and AN

longitudinal sections treated with equal dilution of SS1 antiserum are shown in Fig. 18 E-G. The lack of IGSS signal with preimmune serum (Fig. 18E) and the strong signal with SS1 antiserum (Fig. 18F) indicated that the signal was specifically due to reaction with SS protein. The SS protein was localized in the vascular cylinder and root tip meristem area of sections of both control and AN root sections(Fig. 18F-G). The AN root showed an increased signal in the epidermis and root cap as compared to the control. The difference in the SS protein level between control and AN root was more evident in cross sections. Fig.18 A,C are cross sections taken 0.5 - 1 cm from the root tip and Fig. B,D are taken 0 - 0.5 cm from the root tip. Cross sections from the upper part of the root (i.e. > 0.5 cm from the tip) did not reveal any difference in the level of SS protein between control (Fig.18A) and AN (Fig. 18C) roots. The sections just above the meristematic area showed a slight but significant level of SS induction in the vascular cylinder of the AN root (Fig. 18D) as compared to the control root (Fig. 18B).

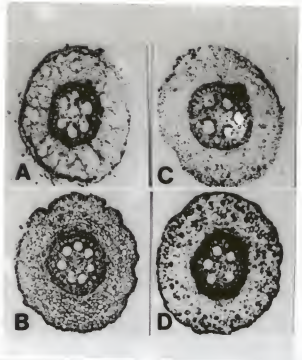
The close-up photographs of control and AN root tips of Sh seedlings stained with preimmune serum and SS1 antiserum are shown in Fig. 19. The root tip stained with preimmune serum showed a very low level of IGSS signal (Fig. 19A). In the root cap region, the AN root showed a higher level of SS protein (Fig.19C) than the control root (Fig. 19B).

Control and AN Sh root sections were also stained with

SS2 antiserum. The IGSS signals with the SS2 antibody in the control root was in the vascular cylinder, pith and epidermis (Fig. 20A). The anaerobic treated root showed a lower level of IGSS signal except in the epidermis and the root cap (Fig. 20B). The close-up photographs of immunolocalization with SS2 antiserum in the Sh root cap of control and AN roots are shown in Fig 20 C and D. A significant increase of the IGSS signal was seen in the root cap of AN seedlings (Fig. 20D).

Because the two SS isozymes are immunologically cross reactive, the Sh deletion mutant sh bz-m4 was used to better define SS2 specificity in the root of maize seedlings. Fig. 21 shows longitudinal and cross sections of control (A,B,F) and AN (C,D,G) sh roots immunostained with SS2 antiserum. A longitudinal section of an AN root treated with preimmune serum is shown in Fig. 21E. The SS2 protein was seen mostly in the vascular cylinder of control roots (Fig. 21F) and in the vascular cylinder and root caps of AN roots (Fig.21G). Fig.21 A,C show cross sections of control and AN roots respectively taken 0.5-1 cm from the root tip. Fig. 21 B,D show cross sections taken through the root caps of control and AN roots. The staining pattern for SS2 protein in the cross sections of control and AN sh roots was similar to that seen in the Sh genotype. The IGSS due to the SS2 antibody reaction increased in the root cap but decreased just slightly in the remainder of the lower root following anaerobic

Fig. 18 A-G. Immunolocalization of SS1 protein in wild-type root sections. A,B- Cross-sections of control roots taken 0.5-1 cm (A) and 0-0.5 cm (B) from the root tip and stained with SS1 antiserum. C,D- Cross section of AN roots taken 0.5-1 cm (C) and 0-0.5 cm (D) from the root tip and stained with SS1 antiserum. E- Longitudinal section of AN root treated with preimmune serum. F,G- Longitudinal sections of control (F) and AN (G) roots stained with SS1 antiserum. All sections were stained with the same dilution of SS1 antiserum. Original maganification x25. VC: vascular cylinder. RC: root cap


A
C
B
D

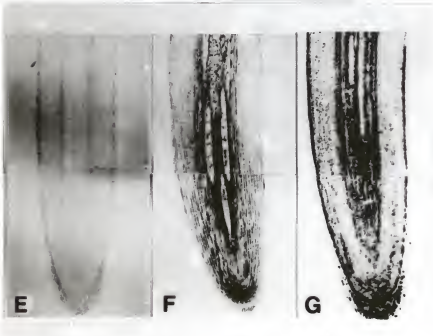

E
F
G

Fig. 19 A-C. Immunolocalization of SS1 protein in Sh root cap. A- Root tip section stained with preimmune serum. B,C- Longitudinal root tip sections of control (B) and AN (C) seedlings stained with SS1 antiserum. Original magnification x31.25 RC: root cap.

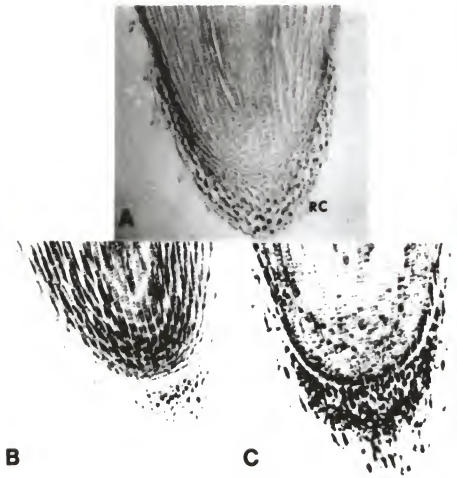

A
RC
B
C

Fig. 20 A-D. Immunolocalization of SS in Sh root section. A,B- Longitudinal sections of control (A) and AN (B) roots stained with the same dilution of SS2 antiserum. C,D- Close up photos of root cap of control (C) and AN (D) roots stained with SS2 antiserum. Original magnification A,B- x25. C,D- 31.25.

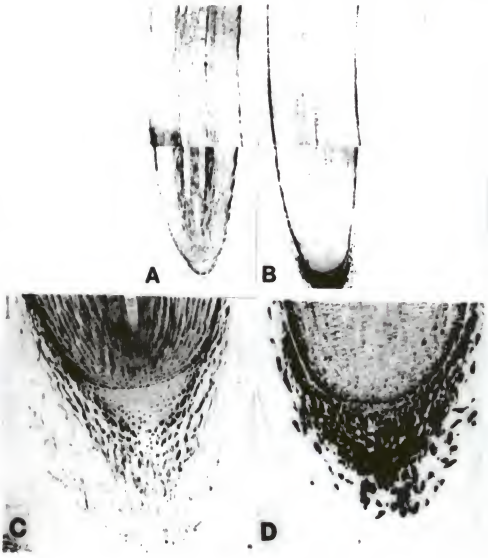
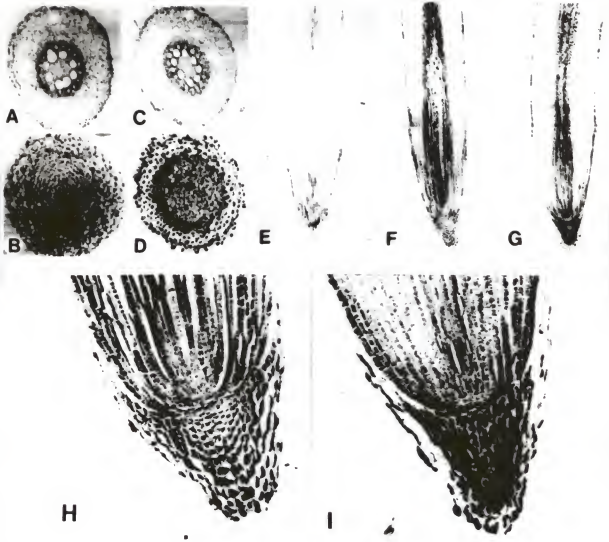

A
B
C
D

Fig. 21 A-I. Immunolocalization of SS2 in sh bz-m4 root sections. A,B- Cross sections taken 0.5-1 cm from the root tip (A) and through the root cap (B) of control roots stained with SS2 antiserum. C,D- Cross-sections taken 0.5-1 cm form the root tip (C) and through the root cap (D) of AN roots stained with SS2 antiserum. E. Longitudinal section of AN root treated with preimmune serum. F,G- Longitudinal sections of control (F) and AN (G) roots stained with the same dilution of SS2 antiserum. Original magnificaiton x25. H,I- Close up photos of root cap of control (H) and AN (I) roots stained with SS2 antiserum. Original magnification x31.25


A
C
B
D
E
F
G
H
I

treatment. Fig. 21 H and I show the close-up photographs of control and AN sh root tip and cap cells stained with SS2 antiserum; the increased intensity of IGSS signal seen in the AN root cap (Fig. 21I) was interpreted as due to AN induction of the Sus gene.

Immunolocalization of SS Protein in Root Cap Cells

Sus induction in root cap cells of Sh and sh genotypes was further analyzed by intracellular immunolocalization of SS2 protein. Electron micrographs of root cap cells of control and AN Sh roots are shown in Fig. 22 A and B respectively. More starch grains per amyloplast were seen in root cap cells of control as compared to the AN root cap cells. More vesicles produced by Golgi apparatus were found in the AN root caps than in the control. However, the sh genotype showed no significant difference between control (Fig. 23A) and AN (Fig. 23B) root cap cells in regard to the number of starch grains in each amyloplast. In the sh genotype less endoplasmic reticulum (ER) was detected in AN than in control root cap cells, and most ER were close to the nucleus or cell membrane in AN root cap cells (Fig. 23 B).

Intracellular immunolocalization of SS protein in control and AN root cap cells of Sh and sh seedlings was done by the protein A gold labeling method and the results are shown in Fig. 24-27. The sections treated with preimmune serum showed very few gold particles due to non-specific staining (Fig. 24A). The gold labeling due to

reaction with SS1 antibody was detected in the cytoplasm but not on the cell wall or amyloplast in control Sh root cap (Fig. 24B). In the AN Sh root cap, the gold labeling was also detected in the cytoplasm (Fig.25 A&B), and the distribution of gold particles was similar to that in the control; however, the density of the signal was slightly higher than in the control root cap.

Immunolocalization of SS2 protein in the control and AN sh root caps is shown in Fig.26 and 27 respectively. The control root cap cell showed a low level of signal in the cytoplasm; a slightly higher density of gold particles was seen around the small amyloplasts (Fig. 26 A&B). In the AN root cap, a random distribution of gold particles was seen in the cytoplasm (Fig. 27A,B).

Fig. 22 A-B. Electron micrograph of Sh root cap cells. A- control. B- AN root. sg: starch grain. G: Golgi apparatus. N: nucleus. MT: mitochondria. CW: cell wall. Bar= 1μ

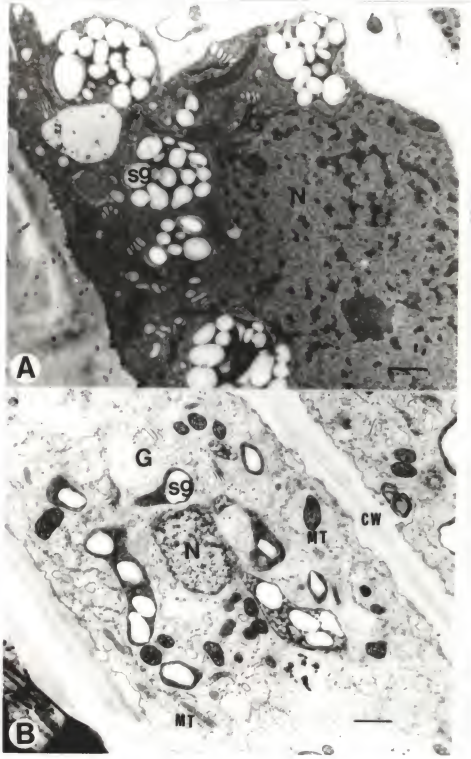

sg
N
A
G
sg
N
MT
CW
MT
B

Fig. 23 A-B. Electron micrographs of sh bz-m4 root cap cells. A- control. B- AN root. sg: starch grain. MT: mitochodria. ER: endoplasmic reticulum. Bar=1μ

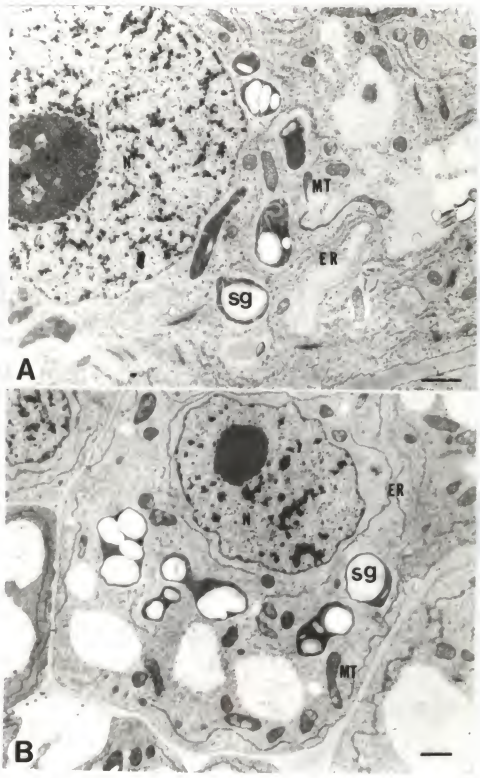

N
MT
ER
sg
A
ER
N
sg
MT
B

Fig. 24 A-B. Electron micrograph showing immunolocalization of SS protein in Sh root cap cell. Thin sections of control root stained with preimmune serum (A) and SS1 antiserum (B). sg: starch grain. CW: cell wall. Bar=0.5μ

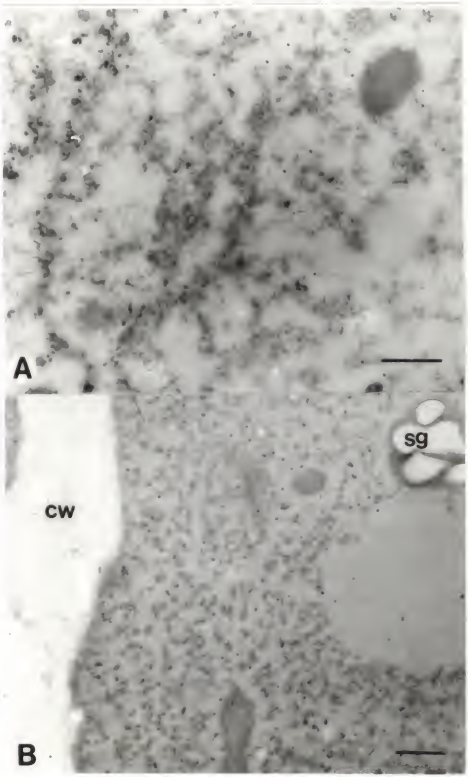

A
sg
cw
B

Fig. 25 A-B. Immunolocalization of SS1 in AN Sh root cap cell. The sections stained with SS1 antiserum. A- showing cell wall and ER. B- showing amyloplast and cytoplasm. CW: cell wall. AP: amyloplast. Bar= 0.5μ

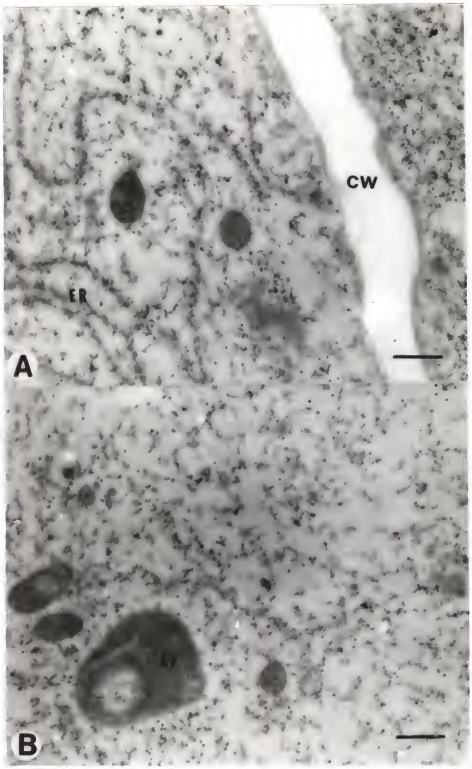

CW
ER
A
B

Fig. 26 A-B. Electron micrographs showing immunolocalization of SS2 in control sh bz-m4 root cap cells. A,B- sections stained with SS2 antiserum. V: vacuole. CW: cell wall. AP: amyloplast. Bar=0.5μ

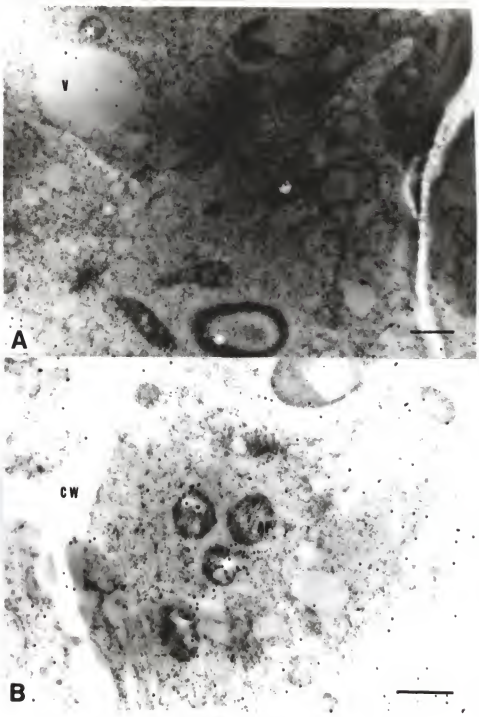
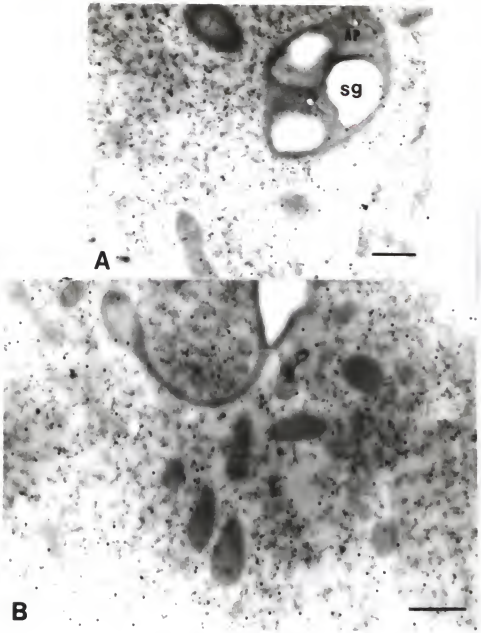

V
A
CW
B

Fig. 27 A-B. Electron micrographs showing immunolocalization of SS2 in AN sh bz-m4 root cap cell. Sections stained with SS2 antiserum. A- showing amyloplast. B- showing cytoplasm of root cap cell. Bar= 0.5μ


sg
A
B

DISCUSSION

Spatial/Temporal Localization of SS Proteins in Kernel

Distribution of SS Proteins in Endosperm

Immunolocalization of SS proteins in various developmental stages of *Sh*, *sh*, and *Sh-r5* kernels demonstrates spatial/temporal differences in distribution of SS proteins. The two very similar sucrose synthase isozymes, SS1 and SS2 (Echt and Chourey 1985; Chourey 1981a) encoded by the *Sh* and *Sus* loci respectively, are differentially located in developing endosperm. In *Sh* kernels, which contain SS1 and SS2, the first cells to accumulate SS1 protein are in the crown region above the embryo. The accumulation of SS1 protein then progresses to the abaxial and the central part of endosperm. The SS1 protein is barely detectable in the basal endosperm transfer cell (BETC) region, and the highest level of SS1 protein is in the crown region (Fig. 2).

The SS2 protein is localized throughout the endosperm with a lower level of immunogold silver (IGS) staining in the *Sh*-deletion mutant which expressed only the SS2 protein. The SS2 protein is also localized in the embryo (Fig. 4). The central crown region where the cavity

eventually forms shows the lowest level of SS2 protein (Fig. 4C).

The Sh-r5 genotype is a Sus-null mutant, and only the SS1 protein is detected in various tissues of the plant (Chourey et al. 1988). The pattern of IGS staining for SS1 protein is similar to that in Sh endosperm. Most SS1 protein is detected at the crown and central region but not at the BETC region (Fig. 12).

Although SS1 and SS2 proteins are immunologically similar and SS1 antibody cross reacts with SS2 antigen and vice versa, the spatial/temporal expression of Sh and Sus genes in endosperm was detected by comparing the distribution of SS proteins in endosperm of these three genotypes.

Location of SS1 Protein Coincides with Starch Accumulation

Since starch and SS1 protein are not transportable intercellularly, the coincidence of I-KI staining and IGSS signal with SS1 antibody identifies individual cells active in the sucrose --> starch conversion reactions. Enzymatic analyses on complementing heterozygotes relative to the sh homozygous parents have previously indicated that the sucrose cleavage role might be the critical physiological function of the SS1 protein (Chourey and Nelson 1979). This is supported by the results presented here.

SS2 Specificity of the BETC and Aleurone Layer

Expression of SS2 in the BETC region was demonstrated by staining a Sh kernel with SS1 and SS2 antibody and a sh mutant with SS2 antibody. The BETC region in the Sh kernel showed no signal with the SS1 antibody but a positive signal with the SS2 antibody. The same level of IGSS signal was detected in the BETC of Sh and sh genotypes when kernels were stained with SS2 antibody (Fig. 8). therefore, it is concluded that the BETC region is SS2-specific.

The BETC are modified in structure to serve for conducting food from the mother plant to the growing endosperm and are characterized by in-growth cell walls, as observed by transmission electron microscopy (Schel et al. 1984; Shannon et al. 1986). The SS2 protein in these conducting cells might be involved in symplastic resynthesis of sucrose from the monosaccharides inverted from sucrose at the base of the kernel. Pulse labeling experiments have demonstrated (Shannon 1972) that labeled glucose and fructose predominate in the lower portion of the endosperm, and the proportion of labeled sucrose becomes greater in the upper parts of the endosperm active in starch synthesis.

The SS2 specificity of the aleurone layer was demonstrated by immunostaining Sh and sh kernels with the same dilution of SS1 antibody. No IGS staining is detectable in the endosperm of the sh kernels except in the

aleurone layer, which shows a high level of staining. The density of IGS staining in the aleurone layer in the sh kernel is similar to that in the Sh kernel (Fig.6), and because there is only SS2 protein present in the sh kernel, the aleurone layer is concluded to be SS2 specific. The results from immunostaining Sh-r5 kernels indicate no detectable IGSS signal in the aleurone layer of the endosperm earlier than 14 DAP. This observation further supports the conclusion that the aleurone layer is SS2 specific.

It is noted that the SS2-specific cells, unlike the cells expressing the SS1 isozyme, are often not associated with starch deposition. The function of SS2 protein in these cells may be different from the SS1 protein in the Sh genotype.

SS2 is Temporally Localized in the Aleurone Layer in Developing Kernels

The SS2 protein in the developing endosperm is first localized in the aleurone layer just above the embryo, then it is progressively localized toward the opposite side of the endosperm. The SS2 signal diminishes gradually in the area where it is seen first during the development of the kernel. The temporal changes of SS2 expression in the aleurone layer is evident by the gradual extinction of the SS2 signal with the advanced maturity of the kernel.

Altered Tissue Specificity of Sh Gene Expression in Sh-r5 Kernel

The Sh-r5 strain exhibits a complete loss of Sus expression in all tissue tested. Furthermore, SS1 protein is detected in the immature embryo which is otherwise characterized by only Sus expression (Chourey et al. 1988).

Immunostaining of Sh-r5 kernels with SS1 antibody shows a positive signal in the embryo at 14 DAP (Fig. 12D). In the Sh and sh kernels stained with SS1 and SS2 antibodies, respectively, SS protein is detected in the embryo as early as 8 DAP (Fig. 2A, 4A). The positive signal in the Sh and sh embryos and the lack of signal in the Sh-r5 embryo prior to 14 DAP confirm the previous conclusion that the embryo is SS2 specific. At 20 DAP, the pattern of SS1 protein in Sh-r5 embryo is similar to that in Sh embryo (Fig. 12E and 2F). The presence of SS1 protein in the Sh-r5 embryo is indicative of the altered tissue specific expression of Sh-r5 allele.

Positive signal is also detected in the aleurone layer near the base of the Sh-r5 kernel (Fig. 12E). Because the aleurone layer is believed to be SS2 specific, the presence of SS1 protein in aleurone layer is also attributed to altered tissue-specific expression of the Sh-r5 allel.

Cavity Formation in the sh Endosperm

During the period between 12 and 16 DAP stages the sh kernel shows specific loss of the central part of the

endosperm (Fig. 4). This period is critical for the occurrence of numerous developmental events in the central endosperm. The most important changes include cell expansion (Kiesselbach 1949), the initiation of probably the most rapid phase of starch deposition (Tsai et al. 1970) and marked increase in nuclear size and DNA content per nucleus (Kowles and Phillips 1985). Because the central endosperm cells in the sh genotype begin to break down just prior to or during these major alterations, the lack of SS1 protein is assumed to interfere with the development of the central endosperm.

Cell degeneration leading to cavity formation in sh endosperm was also examined by scanning and transmission electron microscopy (SEM & TEM). No major difference in the cell wall between the two genotypes was seen by SEM analysis, except that fewer and smaller starch grains were observed in the sh kernel. The discontinuation of the cell wall in sh endosperm prior to cavity formation detected by TEM was not seen in Sh endosperm.

One of the roles of SS1 in maize endosperm is the cleavage of sucrose to UDP-glucose and fructose. UDP-glucose, besides being a substrate for starch biosynthesis, is also the precursor for the synthesis of cell wall polysaccharides, rhammogalacturonan and arabino-gatactan (Goodwin and Mercer 1983; Burgess 1985). When a cell grows, the cell wall extends. Since neither the thickness of the primary wall nor its strength declines as the cell

grows, new material must continually be added to the structure (Albersheim 1975). It is possible that the lack of SS1 protein in sh endosperm may cause a shortage of UDP-glucose for cell wall growth; hence, at the critical stage for cell expansion (12-16 DAP), breakdown of the cells in the center of the endosperm occurs. Another possibility is that the translocated sucrose from the BETC region leads to an osmotic imbalance relating to the lesion in the sucrose to starch reactions.

Intracellular Immunolocalization in Endosperm Cells

Immunolocalization of SS Proteins

SS immunolocalization studies were extended to the TEM level. The results of intracellular immunostaining with SS1 and SS2 antiserum show that the gold labeling of SS1 and SS2 proteins is located in the cytoplasm with no specific localization to starch grains or cell walls (Fig. 15A-B). This agrees with conclusions from previous studies that sucrose synthase is a cytosolic and extravacuolar enzyme (Keller et al. 1988). The lack of heteropolymerization among SS subunits led Chourey et al. (1986) to suggest, among other possibilities, that the SS1 and SS2 subunits were compartmentalized such that subunit interaction was not possible. The data here do not support that hypothesis.

Immunolocalization of Wx Protein

Wx protein, the protein product encoded by the Wx locus, is a starch granule-bound glucosyl transferase expressed in developing maize endosperm (Nelson and Tsai 1964). The sections of kernels treated with Wx antiserum showed, as expected, specific labeling of gold particles on the starch granules, and very little labeling was seen in the other portion of the cell (Fig.14).

Distribution of SS Proteins in Roots of Maize Seedlings

In both Sh and sh roots, immunolocalization studies show that the highest levels of SS protein are in the vascular cylinder, meristem and epidermis. A low level of signal is seen in cortical cells. The unloading of sucrose from sieve tubes and the utilization of sucrose for active cell metabolism in the root apex may explain why SS proteins are located in the vascular cylinder and meristem.

Previous data (Chourey et al. 1986) describing tissue-specific polymerization of the SS1 and SS2 subunits in the Sh genotype shows that there are five SS tetramers, including two homotetramers and three heterotetramers, in the root extracts. Data obtained in this study indicate that there was no cell-specific immuno-histological difference between Sh and sh genotypes. Therefore, it is concluded that both genes are expressed simultaneously in the same cells. Results from intracellular localization of

SS proteins in root of Sh and sh genotypes further support this conclusion.

SS Proteins Are Induced in Root Tip of AN Seedling

Previously, the increase in the steady state level of SS1 transcript in AN seedlings was shown to be unassociated with an increase in the SS1 protein level (McElfresh and Chourey 1988). This conclusion relied on Northern and Western blot data and was based on average RNA and protein levels measured from whole root extracts. The results from immunolocalization of SS1 and SS2 proteins in roots of aerobic and anaerobic treated maize seedlings constitute the first detailed demonstration of the expression of Sh and Sus genes at the cellular level.

SS1 Protein Is Induced in the Lower Approximately 1 cm of the Root

Immunolocalization studies using SS1 antibody on longitudinal root sections do not show significant differences between control and AN samples, but cross sections taken within 1 cm of the root tip do show a slight but consistent induction of SS1 protein following anaerobic treatment (Fig. 18). The level of SS1 protein increases in the vascular cylinder, pith and epidermis in the AN root tip (Fig. 18 B-D). Cross sections taken 1-2 cm from the root tip, however, do not show any SS1 induction.

During anaerobiosis, the increased rate of glycolysis

would create a higher demand for hexose sugars, especially in the rapidly dividing cells of the lower root. The higher level of SS1 in the vascular cylinder of anaerobic roots might meet this demand by cleaving more sucrose molecules.

SS2 Protein is Induced in Root Caps of AN Seedlings

Results from immunolocalization experiments using wild-type and sh root sections indicate that in AN roots the SS2 protein level decreases slightly throughout the lower root, with the major exception of the root cap where the SS2 protein level increases. As in situ hybridization studies indicate that the SS2 RNA level decreases in the lower root, including root cap, in response to anaerobiosis (Rowland et al. 1989), the rise of SS2 protein in the root cap may reflect post-transcriptional regulation. The SS2 message present in control roots may be rapidly translated early during anaerobiosis and then degraded.

The root apex, including the tip and cap, is physiologically an important utilization sink for sucrose due to several critical metabolic activities in this region. Saglio (1985) has observed impaired transport of sucrose to the root tip area when the entire root is made anoxic. Sucrose depletion in excised maize root tips has been shown to induce the SS1 transcripts (Koch and McCarty 1988). Consequently, the effect of anaerobic stress on sucrose synthase levels in the root tip could be more

directly due to depletion of sucrose than to lack of oxygen. Data are not available to distinguish between these possibilities at this time.

Root Cap Cells of Control and AN Seedlings

Immunolocalization of SS Proteins in Root Cap Cells

The higher levels of SS protein in AN root cap cells were further examined by electron microscopy. The localization of SS proteins in the root cap cells is similar to that in endosperm cells. The SS proteins are in the cytoplasm and not specifically bound to cell wall or starch grains. A higher density of gold labeling is seen in the AN root cap cells of Sh and sh seedlings although no detailed quantitative analysis was perfomed.

Ultrastructure of Root Cap Cells

Several differences are found between control and AN root cap cells. Fewer starch grains in the amyloplasts, swollen Golgi complexes and more vesicles are found in AN Sh root cap cells. In sh root cap cells, no difference is seen in the Golgi complexes or in the number of starch grains per amyloplast between control and AN root. However, the endoplasmic reticulum is found around the nucleus and cell membrane of AN root cap cells (Fig. 23). Physiological significance of the reduction in the number of starch grains is not clear.

The ultrastructure of root meristems of AN maize seedlings have been described by Aldrich et al. (1985). An anaerobiosis treatment for less than 18 hr results in larger cells, larger perhaps polyploid nuclei, swollen mitochondria, and increased production of Golgi vesicles. After that time, mitochondria, nuclei, and vacuoles resume near-normal dimensions. The AN effects on the ultrastructure of meristematic cells were not observed in the root cap cells shown here except for swollen Golgi vesicles.

CONCLUSIONS

The two sucrose synthase isozymes, SS1 and SS2, are differentially localized in developing kernels of maize. SS1 is localized in the crown and central regions of the endosperm, and accumulation of SS1 coincides with starch deposition. SS2 is localized in the embryo, aleurone layer and basal endosperm transfer cells; the presence of SS2 in these cells is not associated with starch accumulation. A low level of SS2 accumulation is observed throughout the storage cells of the sh endosperm; however, the presence of SS2 in the storage cells of the Sh endosperm cannot be confirmed. The data show the spatial/temporal expression of the two sucrose synthase genes in the endosperm of maize.

The pattern of intracellular localization of SS1 and SS2 are similar. SS proteins are localized in the cytoplasm without specific localization to amyloplasts, cell walls, or other organelles. This indicates that there is no intracellular compartmentalization of the SS1 and SS2 subunits in endosperm cells.

In the root, both SS1 and SS2 are localized mainly in the vascular cylinder and in the epidermal cells. Because there is no cell-specific localization of SS1 and SS2 and

because of the previous demonstration of SS heterotetramers in root extracts, it is concluded that both Sh and Sus genes are expressed simultaneously in the same cells. Immunolocalization of SS proteins in aerobic and anaerobically stressed (AN) maize seedlings indicates that SS1 is induced only in the lower part close to the apex of AN roots including root caps, and the SS2 is induced in root caps of AN seedlings.

APPENDIX

FAA fixative

95% EtOH	50ml
Glacial Acetic Acid	5ml
37% Formaldehyde	10ml
Deionized water	35ml

TBA dehydration series

Step	95% EtOH	TBA	H2O	time needed
	ml	ml	ml	day
I	40	10	50	1
II	50	20	30	1
III	50	35	15	1
IV	45	55	0	1.5
V	25*	75	0	1.5
VI	0	100	0	1
VII	0	100 (Safranin O added)		1
VIII	0	100	0	1

* : 100% EtOH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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